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THEREOF

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Abstract 1

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[Title of the Invention] HUMAN p51 GENE AND GENE PRODUCT
THEREOF

[Claims]



[Claim 1] A gene coding for the following
protein (a) or (b):

(a) a protein having an amino acid sequence shown in Fig.
1;

(b) a protein having an amino acid sequence derived from
10 the amino acid sequence shown in Fig. 1 by deletion,
substitution or addition of one or a plurality of amino
acids and having p51 activity.

[Claim 2] A gene comprising the following DNA

(a) or (b):

15 (a) a DNA having a nucleotide sequence identified by the
nucleotide numbers 145-1487 of the nucleotide sequence
shown in Figs. 2-8;

(b) a DNA capable of hybridizing with a DNA having a
nucleotide sequence identified by the nucleotide numbers
20 145-1487 of the nucleotide sequence shown in Figs. 2-8
under stringent conditions and coding for a protein having
p51 activity.

[Claim 3] The gene according to claim 1 or 2
which has the nucleotide sequence shown in Figs. 2-8.

25 [Claim 4] A cDNA comprising the following DNA

(a) or (b):

(a) a DNA having a nucleotide sequence identified by the nucleotide numbers 145-1487 of the nucleotide sequence shown in Figs. 2-8;

5 (b) a DNA capable of hybridizing with a DNA having a nucleotide sequence identified by the nucleotide numbers 145-1487 of the nucleotide sequence shown in Figs. 2-8 under stringent conditions and coding for a protein having p51 activity.

10 [Claim 5] A DNA characterized in that it is capable of the hybridizing with a DNA having a nucleotide sequence identified by the nucleotide numbers 145-1487 of the nucleotide sequence shown in Figs. 2-8 under stringent conditions.

15 [Claim 6] A vector harboring the gene according to any one of claims 1-4.

[Claim 7] A host cell transformed with the vector according to claim 6.

[Claim 8] A protein defined below in (a) or
20 (b):
(a) a protein having an amino acid sequence shown in Fig. 1;
(b) a protein having an amino acid sequence derived from the amino acid sequence shown in Fig. 1 by deletion,
25 substitution or addition of one or a plurality of amino

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acids and having p51 activity.

[Claim 9] A method for producing the protein according to claim 8, which comprises growing the host cell according to claim 7 in a culture medium and
5 harvesting a protein from the resulting culture.

[Detailed Description of the Invention]

[0001]

[Technical Field to Which the Invention Pertains]

The present invention relates to a novel human
10 gene analogous to human p53 and human p73 genes, which are
known as tumor suppressor genes, and the corresponding
gene product.

[0002]

[Prior Art]

15 The p53 protein was discovered as a nuclear
protein binding to the large T antigen of the DNA tumor
virus SV40 and its gene has been cloned. At first, the
p53 gene was considered to be an oncogene because the
transfer of this gene and the ras gene together into cells
20 resulted in transformation of embryonal cells. Later
studies, however, revealed that the initially cloned p53
gene was a mutant type and that the wild type rather
suppressed the transforming activity of the mutant type.
By now, deletions or anomalies in the p53 gene have been
25 detected in many human cancers and a gamate mutation of

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the p53 gene was also discovered in Li-Fraumeni syndrome which is known to be a hereditary disease with a high risk for malignant conversion. Because of these findings, the p53 gene has by now been considered to be an important
5 suppressor oncogene [Baker, S. J., et al., Science, 244, 217-221 (1989); Nigro, J. M., Nature, 342, 705-708 (1989)].

[0003]

The human p53 protein consists of 393 amino acid residues and can be roughly divided into the N-terminal
10 domain (the 1-101st amino acid region), the core domain (the 102-292nd amino acid region), and the C-terminal domain (the 293-393rd amino acid region). The N-terminal domain contains sequences necessary for transcriptional regulation, such as acidic amino acids and a high-proline
15 region, and is considered to be a transcriptional activator domain. The central core domain contains 3 hydrophobic sites and is a domain associated with nucleotide sequence-specific DNA binding. The C-terminal domain contains many basic amino acids and a sequence
20 necessary for tetramerization and is considered to be responsible for recognition of nonspecific DNA binding and DNA damage and inhibition of transformation.

[0004]

Many of the p53 gene abnormalities detected in
25 human cancer cells are missense mutations and most of them

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are concentrated in the core domain corresponding to the 100~300th amino acid sequence from the N terminus, particularly in the region called "hot-spot" which has been conserved among species. The hot-spot region in the
5 core domain is the sequence associated with the binding between p53 protein and DNA and, actually, mutation of this region results in the inhibition of specific binding to DNA.

[0005]

10 It became clear from the above that the p53 protein plays the role of a transcriptional control factor which binds specifically to other genes to modulate expression of the genes.

[0006]

15 The gene whose transcription is induced by the p53 protein includes, among others, the p21 gene [known as WAF1, CIP1, or SDI1 (El-Dairy, W. S., et al., Cell, 75, 817 (1993)); MDM2 (Wu. X., et al., Genes Dev., 7, 1126 (1993)); MCK (Weintraub. H., et al., Proc. Natl. Acad. Sci.
20 USA, 88, 4570 (1991); Zambetti. G. P., et al., Genes Dev., 6, 1143 (1992))], GADD45 [Kastan, M. B., et al., Cell, 71, 587 (1992)], Cyclin G [Cyclin G: Okamoto, K., EMBO J., 13, 4816 (1994)], BAX [Miyashita, T., et al., Cell, 80, 293 (1995)], and insulin-like growth factor-binding protein 3
25 [IGF-BP3: Buckbinder, L., et al., Nature, 377, 646 (1995)].

[0007]

The protein encoded by the p21 gene is an inhibitor protein for cyclin-dependent kinase (CDK), and it has been found that the wild type p53 protein regulates the cell cycle in an inhibitory way through p21 [Harper, J. W., et al., Cell, 75, 805 (1993); Xiong, Y., et al., Nature, 366, 707 (1993); Gu, Y., et al., Nature, 366, 701 (1993)]. Furthermore, the p21 gene reportedly binds to the proliferating cell nuclear antigen (PCNA) to directly inhibit DNA replication [Waga, S., et al., Nature, 369, 574 (1994)]. In addition, the p21 gene has been found to the same gene as the SDI1 gene which induces senescence of cells to inhibit DNA synthesis [Noda., A., et al., Exp. Cell Res., 211, 90 (1994)].

15 [0008]

MDM2 binds to the p53 protein to inactivate the transcriptional regulation activity of the gene protein, leading to the putative conclusion that MDM2 is acting as a negative feedback regulating factor.

20 [0009]

IGF-BP3 is a negative regulating factor in IGF signalization. Therefore, the increase of the IGF-BP3 gene by the p53 protein suggests the possible outcome that the p53 protein induces suppression of growth of IGF-dependent cells.

25

[0010]

Meanwhile, the wild type p53 protein reportedly induces apoptosis of myelocytic leukemia cells [Yonish-Rouach, E., et al., Nature, 352, 345 (1991)]. Induction
5 of thymocyte apoptosis by irradiation does not take place in p53-defective mice [Lowe, S. W., Nature, 362, 847 (1993); Clarke, A. R., et al., Nature 362, 849 (1993)] and, in the crystalline lens, retina and brain, the p53 protein induces apoptic death of cells deprived of normal retinal
10 blastoma gene (RB gene) activity [Pan, H., and Griep, A. E., Genes Dev., 8, 1285 (1994); Morgenbesser, S. D., et al., Nature 371, 72 (1994); Howes, K. A., Genes Dev., 8, 1300 (1994); Symonds, H., et al., Cell, 78, 703 (1994)]. E. White proposes that the p53 protein is useful for a
15 surveillance of RB gene mutation and that the protein is likely to induce apoptosis of the cells in which a RB gene mutation is involved [White, E., Nature, 371, 21 (1994)].

[0011]

Furthermore, in the mouse erythroid leukemia
20 cell line in which the temperature-sensitive p53 gene only is expressed, a fall in temperature results in reconversion of the mutant p53 gene to the wild type to induce apoptosis and the mutant p53 gene isolated therefrom imparts the ability to grow in soft agar medium
25 to a p53-defective fibroblast line (impart anchorage

independence) [Xu et al., Jpn, J. Cancer Res. 86: 284-291 (1995); Kato et al., Int. J. Oncol. 9: 269-277].

[0012]

BAX is able to bind to bcl-2, which is an
5 inhibitor of apoptosis, and encourages apoptic cell death
[Oltvai, Z. M., et al., Cell, 74, 609 (1993)]. The
increase in the BAX gene and decrease in bcl-2 by the p53
protein are involved in the apoptosis of the mouse
leukemia cell line M1 [Miyashita, T., et al., Oncogene, 9,
10 1799 (1994)] and Fas, which is one of the signal
transducers for apoptosis, is increased in non-small-cell
lung cancer and erythroleukemia [Owen-Schaub, L. B., et
al., Mol. Cell Biol., 15, 3032 (1995)].

[0013]

15 The many investigations referred to above have
revealed that the p53 protein either activates or
represses the transcription of various genes not limited
to the p21 gene. Moreover, even the mutant p53 protein
defected in the transcriptional regulating function is
20 capable of interacting with other intracellular proteins
to transmit signals and discharge a DNA damage repairing
function.

[0014]

Functions of the p53 protein which have so far
25 been identified are a transcription regulating function, a

signal transducer function through binding to other intracellular proteins, a constituent element of a protein complex related to DNA replication, a DNA binding function, and exonuclease activity. Interplay of these functions consequently causes the arrest of the cell cycle in cells, induction of apoptosis, DNA repair, regulation of DNA replication, and induction of differentiation.

[0015]

Furthermore, the functions of the p53 protein are not expressed only in the event of a gene damage and it is reported that when the living tissue is subjected to various stresses such as viral infection, cytokine stimulation, hypoxia, a change in the nucleotide pool, drug-induced metabolic abnormality, etc., the stimuli trigger quantitative or qualitative changes in the p53 protein. The p53 protein subjected to the quantitative or qualitative regulation expresses its functions, such as signal transduction through interactions with other proteins and control of the transcription of other genes, to regulate the replication of DNA in cells of the living tissue subjected to biological stresses, repair the cells by suspending the cell cycle, eliminate cells by way of apoptosis, or promote the differentiation of cells, thereby contributing to the protection of the living tissue against the stresses [Ganman, C. E., et al., Genes

Dev., 2, 600-611 (1995): Graeber, T. G., et al., Nature, 372, 88-91 (1996): Linke, S.P., et al., Genes Dev., 10, 934-947 (1996): Xiang, H., et al., J. Neurosci., 16, 6753-6765 (1996)].

5 [0016]

In view of the existence of p53 gene mutations in a half of human tumors, clinical application of the p53 gene and its product protein to the diagnosis and therapy of tumors has been a subject of study in recent years.

10 The method of detecting tumor cells invading the lymph node or body fluid by carrying out a PCR using primers specifically recognizing the mutation site of the p53 gene can be an effective diagnostic technique for estimating the scope of tumor invasion or predicting a recurrence of
15 the tumor [Hayashi, H., et al., Lancet, 345, 1257-1259 (1995)].

[0017]

Furthermore, taking advantage of the apoptosis-inducing activity of the p53 protein, a gene therapy
20 comprising introducing a wild type p53 gene into the tumor cell by means of a virus vector is being practiced in the United States and its effectiveness has been reported [Roth, J. A., et al., Nature Med., 2, 985-991 (1996)].

[0018]

25 Meanwhile, more than the majority of human

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tumors are not associated with p53 gene mutation and, from this fact, the possibility of existence of other tumorigenesis-inhibitory proteins analogous to the p53 protein has been pointed out. The inventors of the present invention previously found that a p53 gene mutation cannot be a useful premonitory indicator of non-Hodgkin's lymphoma (NHL).

[0019]

Recently, a novel gene, named p73, which has high homology to said p53 gene has been identified [Kaghad, M., et al., Cell, 90, 809-819 (1997)]. According to the information available to the present inventors, the p73 protein shows 29% homology to the human p53 protein in the transcriptional activator domain (the 1st-45th amino acid region). Moreover, this p73 protein has a homology of 63% in the DNA binding domain (the 113rd ~ 290th amino acid region) having 6 complementary conserved sequences called hot spots of mutation; and a homology of 38% in the oligomerization domain (the 319th ~ 363rd amino acid region). With regard to the C-terminal domain, however, no significant homology has been recognized between p73 protein and p53 protein.

[0020]

It is reported that excessive expression of the
25 p73 protein inhibits the growth of a neuroblastoma cell

line and SAOS2 cells (an osteosarcoma cell line) and that
a transient expression of the p73 protein promotes the
apoptosis of SAOS2 cells and baby hamster's kidney cells
[Bruce Clurman and Mark Groudine, Nature, 389, 122-123
5 (1997); Christine, A., et al., Nature, 389, 191-194
(1997)].

[0021]

However, the p73 protein is somewhat different
from the p53 protein in that the former is expressed only
10 at low levels in normal tissues. Moreover, the p73
protein is different from the p53 protein in that the
expression of the former protein in a neuroblastoma cell
line is not induced by UV irradiation or a low dose of
actinomycin D.

15 [0022]

Therefore, it is not true that the p73 protein
has the exactly the same functions as those of the p53
protein and, at the present, much depends on further
research. There is a report arguing that, based on the
20 observations so far made, this p73 may be categorized as a
putative tumor suppressive factor in neuroblastoma.

[0023]

[Problem to be Solved by the Invention]

It is an object of the present invention to
25 provide information on a novel gene related to the

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morphogenesis of human tumors and the corresponding gene product. It is a further object of the present invention to provide a partial DNA of the gene which is useful as a primer and a probe, a vector harboring said gene, a transformant transformed by introducing said vector, and a method for producing said gene product which comprises growing said transformant.

[0024]

The gene of the present invention is a gene analogous to the p53 gene which is known as a tumor suppressor gene. The gene is expected to achieve the following matters. Using the gene, one can analyze the expression level and functions of various cells, and the analysis of the expression product further enables the detection of a cause of disease, diagnosis and treatment of the disease (for example, malignant tumor, etc.).

[0025]

[Means for Solving the Problem]

Since more than the majority of human tumor
20 tissues do not have mutants of the p53 gene as mentioned
above, the possibility has been suggested of the existence
of other gene products than the p53 protein, which have
the tumorigenesis-inhibiting function.

[0026]

25 Therefore, the inventors of the present

invention did intensive investigations in search for novel genes which might be associated with said tumorigenesis-inhibiting function. As a result, they discovered a novel human gene coding for a protein showing activity similar
5 to that of said p53 protein and confirmed that the particular gene or the gene product is significantly associated with apoptosis. The present invention has been accomplished based on this finding.

[0027]

10 The present invention, therefore, is directed to human p51 genes described below in (1) to (3) and the related genes.

[0028]

(1) A gene coding for the following protein (a) or (b):

15 (a) a protein having an amino acid sequence shown in Fig. 1;

(b) a protein having an amino acid sequence derived from the amino acid sequence shown in Fig. 1 by deletion, substitution or addition of one or a plurality of amino
20 acids and having p51 activity.

[0029]

(2) A gene comprising the following DNA (a) or (b):

(a) a DNA having a nucleotide sequence identified by the nucleotide numbers 145-1487 of the nucleotide sequence
25 shown in Figs. 2-8:

5 [0035]

(6) A protein described below in (a) or (b):

10 (a) a protein having an amino acid sequence shown in Fig.
1;

[0037]

20 [0038]

25 [0039]

It should be understood that the designation of "p51" is used only for convenience's sake in this specification and is by no means definitive of the gene and protein of the present invention.

5 [0040]

Furthermore, the term "gene (DNA)" in the context of the present invention means not only a double-stranded DNA but also a single-stranded DNA, inclusive of the component sense chain and antisense chain, and is by
10 no means limitative of its length. Therefore, unless otherwise indicated, the gene (DNA) of the present invention includes the double-stranded DNA inclusive of human genomic DNA, a single-stranded DNA comprising the cDNA (sense chain), a single-stranded DNA having a
15 sequence complementary to said sense chain (antisense chain), and all fragments thereof.

[0041]

The representation of amino acids, peptides, nucleotide sequences, nucleic acids, etc. by abbreviations
20 in this specification conforms to the recommendations by IUPAC-IUB, the "Guidelines for Preparation of Specifications, etc. which contain Nucleotide Sequences or Amino Acid Sequences" (edited by the Japanese Patent Office), and the conventions in the use of symbols in the
25 related field of art.

[0042]

[Mode for Carrying out the Invention]

The present invention relates to novel human genes coding for proteins having p51 actions identical or
5 equivalent to the actions or functions of the p53 protein.

[0043]

The gene according to the present invention has been acquired by carrying out a PCR using primers newly established after energetic explorations using specific
10 regions selected from the sequences of the hitherto-known p53 gene and p73 gene with judicious endeavors. More particularly, by carrying out a PCR using the above established primers, a gene fragment which is not identical but similar to the p53 gene and p73 gene was
15 obtained. By using this DNA fragment as a probe, a cDNA clone coding for a novel protein having high homology to the amino acid sequence of p53 protein was successfully isolated from among cDNA clones randomly selected from a human skeletal muscle cDNA library.

20 [0044]

The calculated molecular mass of the amino acid sequence deducted from the cDNA thus obtained was about 50,894 Da and, therefore, the present inventors named this cDNA "human p51 gene" and the protein having the amino
25 acid sequence encoded by this gene "p51 or p51 protein"

(or "p51 protein") for convenience's sake.

[0045]

With respect to the amino acid sequence in each domain of the p51 protein encoded by the gene of the invention, the homology to the corresponding domain of the known proteins p53 or p73 was investigated with FASTA Program using the GCG software (Wisconsin Sequencing Package, Genetics Computer Group) [Person, W. R. and Lipman, D. J., Proc. Natl. Acad. Sci. U.S.A., 85, 1435-10 1441 (1988)]. The results are shown in Table 1 (cf. Figs. 1 and 2). For reference, the homologies between p53 protein and p73 protein as determined by the same method are also shown in Table 1.

[0046]

15 [Table 1]

An investigation of the transcription products of p51 genes in various human tissues revealed that the gene has alternative splicing variants, namely, the expression products exist as spliced in a short form 20 (p51A) and a long form (p51B) encoding p51 (see Fig. 12).

[0047]

The p51 gene product of the present invention shows transcriptional activation activity, cell growth-inhibitory activity and apoptosis-inducing activity which 25 are similar to the activities of the p53 protein.

Furthermore, the expression of the p51 gene in human tissues was found to be more tissue-specific than the expression of the p53 gene and, compared with the expression of the p73 gene which is also tissue-specific, was broader in tissue distribution although there was an overlap of expression pattern between them.

[0048]

Further, by using radiation hybrid cells, it was revealed that the p51 gene locally exists in the 3q26 region of a human chromosome, which is a domain relating to the morphogenesis of NHL (non-Hodgkin's lymphoma) and other tumors. Moreover, the mutation of the p51 gene was found in the human tumor tissues or tumor cell lines.

[0049]

The above findings suggested strongly that the human p51 gene of the present invention is a new member of the p53 tumor suppressor gene family.

[0050]

As a specific example of the gene according to the present invention, there can be mentioned one having the DNA sequence possessed by the clones described in Example 1 which appears hereinafter.

[0051]

The gene possessed by the clone has an open reading frame of 1344 nucleotides encoding a protein

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consisting of 448 amino acid residues shown in Fig. 1 (the nucleotide sequence identified by the nucleotide numbers 145~1487 of the nucleotide sequence shown in Figs. 2-8).

[0052]

5 The full-length nucleotide sequence of the cDNA clone consists of 2816 nucleotides as shown in Figs. 2-8. In the nucleotide sequence shown in Figs. 2-8, the initiation codon (ATG) is situated in the 145~147 nucleotide position and the polyadenylation signal (AATAA)
10 is situated in the 2786~2791 position.

[0053]

 The p51 protein of the invention having 448 amino acids encoded by the p51 gene of the invention has a transcriptional activation domain corresponding to the
15 amino acid numbers 1~59, a DNA binding domain corresponding to the amino acid numbers 142~321, and an oligomerization domain corresponding to the amino acid numbers 353~397, as shown in Fig. 1.

[0054]

20 Thus, the p51 gene according to the present invention includes a human p51 gene having a nucleotide sequence coding for a protein having the amino acid sequence shown in Fig. 1. However, the p51 gene of the invention is not limited to such gene but includes all
25 homologs of the human p51 gene.

[0055]

The term "homolog of human p51 gene" means any member of a group of interrelated genes, which are analogous to the p51 gene of the invention and/or gene products in nucleotide sequence, have common structural features and a common gene expression pattern and are analogous in the biological functions as mentioned above and therefore regarded as constituting one gene family. In this sense, alleles of the human p51 gene are, of course, subsumed in the concept of said "homolog".

[0056]

As an example of said homolog, there can be mentioned a gene which codes for a protein having an amino acid sequence resulting from the mutation or modification of one or a plurality of sites of the amino acid sequence shown in Fig. 1 and having actions similar to those of said p51 protein having the amino acid sequence shown in Fig. 1.

[0057]

The degree of homology in amino acid sequence may generally be not less than about 45%, preferably not less than about 50%, in terms of the full-length sequence as determined with FASTA Program using GCG software (Wisconsin Sequencing Package, Genetics Computer Group) (Person, W. R. and Lipman, D. J., Proc. Natl. Acad. Sci.

U.S.A., 85, 1435-1441 (1988)]. Preferably, the homology should fall in at least one of the following range: the homology in the transcriptional activation domain is about 35% or higher, preferably not less than 45%, the homology in the DNA binding domain is 88% or higher, preferably not less than about 90%, and the homology in the oligomerization domain is about 70% or higher, preferably not less than about 80%.

[0058]

10 Thus, the gene of the present invention includes any gene having a nucleotide sequence coding for a protein having an amino acid sequence derived from the sequence shown in Fig. 1 by deletion, substitution or addition of one - a few or a plurality of amino acids.

15 [0059]

The extent of "deletion, substitution or addition of amino acids" and the site or sites involved are not particularly restricted inasmuch as the protein so modified is functionally equivalent to the protein (p51 protein) having the amino acid sequence shown in Fig. 1. Thus, the term "p51 activity" as used in this specification means the activities and functions possessed by the protein having the amino acid sequence shown in Fig. 1, thus including tumor cell growth inhibitory activity, apoptosis-inducing activity and transcriptional regulation

20

25

As the functions and actions of the p53 protein, there may be mentioned a transcriptional regulation function, a signal transduction function which is expressed through its binding to other intracellular proteins, the function as a component of the protein complex related to DNA replication, a DNA binding function, exonuclease activity, etc., and as the functions expressed by the composite interplay of said various functions, a cell cycle interrupting function, an apoptosis-inducing function, a DNA repairing function, a DNA replication control function and/or a differentiation-inducing function in cells. It is considered that the p51 protein of the present invention has some or all of these functions and actions.

[0062]

The modification (mutation) of an amino acid sequence may be spontaneous, e.g. spontaneous mutation or posttranslational modification, but can be artificially
5 induced on the basis of a native gene (e.g., human p51 gene of the invention). The present invention encompasses all modified genes coding for proteins having the above-mentioned characteristics without regard to the cause or means of mutation or modification. In this sense, the
10 gene of the invention (e.g., human p51 gene of the invention) includes alleles of the gene coding for the protein having the amino acid sequence shown in Fig. 1.

[0063]

The means for making such artificial
15 modifications includes genetic engineering techniques such as site-specific mutagenesis [Methods in Enzymology, 154: 350, 367-382 (1987); ditto 100: 468 (1983); Nucleic Acids Res., 12: 9441 (1984); Zoku Seikagaku Jikken Koza 1 "Idenshi Kenkyuho II" [Experimental Biochemistry Series 1
20 "Methods for Gene Research II" (edited by Japanese Biochemical Society), p105 (1986)], etc. and chemical synthetic techniques such as the phosphotriester method and the phosphoamidate method [J. Am. Chem. Soc., 89: 4801 (1967); ditto 91: 3350 (1968); Science, 150: 178 (1968);
25 Tetrahedron Lett., 22: 1859 (1981); ditto 24: 245 (1983)]

as well as a suitable combination of such techniques.
More specifically, DNA synthesis can be carried out
chemically by the phosphoramidite method or the triester
method, or on a commercial automatic oligonucleotide
5 synthesizer. The double-stranded chain fragment can be
obtained by synthesizing complementary chains and
annealing them together under suitable conditions or can
be obtained from a chemically synthesized single-stranded
chain by adding a complementary chain using a DNA
10 polymerase together with suitable primer sequences.

[0064]

As a specific example of the gene of the
invention, there can be mentioned the gene having a
nucleotide sequence corresponding to the nucleotide
15 numbers 145-1487 of the nucleotide sequence shown in Figs.
2-8. Each of these nucleotide sequences represents an
example of combination of the codons coding for the
respective amino acid residues of the amino acid sequence
shown in Fig. 1. Therefore, the gene of the present
20 invention is not limited to genes having such specific
nucleotide sequences but may have nucleotide sequences
designed by using a combination of optional codons for
each amino acid residue. Selection of codons can be made
in the routine manner, for example with reference to the
25 frequency of utilization of each codon by the host to be

used [Nucleic Acids Res., 9, 43 (1981)].

[0065]

Furthermore, as mentioned above, the gene of the present invention includes a nucleotide sequence having a defined degree of homology to the nucleotide sequence corresponding to the nucleotide numbers 145~1487 of the nucleotide sequence shown in Figs. 2-8 [hereinafter sometimes referred to briefly as "(145-1487) in Figs. 2-8"].

10 [0066]

As an example of such gene, there can be mentioned a gene having a nucleotide sequence capable of hybridizing with a DNA having said nucleotide sequence (145-1487) in Figs. 2-8 under stringent conditions, for example in 0.1% SDS-containing 0.2 x SSC at 50°C or in 0.1% SDS-containing 1 x SSC at 60°C.

[0067]

The gene of the present invention can be easily produced and acquired by the standard genetic engineering techniques [Molecular Cloning 2d Ed, Cold Spring Harbor Lab. Press (1989); Zoku Seikagaku Jikken Koza 1 "Idenshi Kenkyuho I, II, III" [Supplemental Biochemical Experimental Series 1 "Methods for Gene Research I, II, III" (edited by Japanese Biochemical Society), (1986), etc.], based on the sequence information on the specific

examples of the gene of the invention disclosed herein.

[0068]

More particularly, the desired gene can be
acquired by constructing a cDNA library from a suitable
5 source in which the gene of the invention can be expressed
and selecting the desired clone from this cDNA library
using a suitable probe or antibody specific to the gene of
the invention in the per se known manner [Proc. Natl. Acad.
Sci., USA., 78: 6613 (1981); Science, 222: 778 (1983),
10 etc.].

[0069]

In the above procedure, the cDNA source includes
but is not limited to various cells or tissues in which
the gene of the invention is expressed and cultured cells
15 derived therefrom. Isolation of the whole RNA from such a
source, isolation and purification of mRNA, acquisition of
cDNA, and cloning thereof can all be carried out in the
routine manner. cDNA libraries are also commercially
available. In the practice of the present invention, such
20 commercial cDNA libraries, for example those available
from Clontech Lab. Inc., can also be employed.

[0070]

The method of screening for the gene of the
invention from a cDNA library is not particularly
25 restricted, either, but the conventional methods can be

selectively employed.

[0071]

To be specific, selection of a cDNA clone by an immunoscreening technique using a specific antibody
5 against the protein produced by the cDNA, the plaque hybridization or colony hybridization technique using a probe having a selective binding affinity for the objective DNA sequence, or a combination thereof can be mentioned by way of example.

10 [0072]

As to the probe to be used in the above procedure, it is generally advantageous to use a DNA chemically synthesized according to the nucleotide sequence information on the gene of the present invention
15 but the very gene of the invention which has already been acquired or a fragment thereof can of course be used with advantage as said probe. Furthermore, the sense primer and antisense primer established based on the nucleotide sequence information on the gene of the present invention
20 can be used as the screening probe.

[0073]

For acquisition of the gene of the invention, DNA/RNA amplification by the PCR method [Science, 230, 1350 (1985)] can also be used with advantage.
25 Particularly under circumstances where a full-length cDNA

can hardly be obtained from a library, the RACE [rapid amplification of cDNA ends] method [Jikken Igaku (Experimental Medicine), 12(6): 35 (1994)], in particular the 5'-RACE method [M. A. Frohman, et al., Proc. Natl. Acad. Sci., USA., 8: 8998 (1988)], can be used with advantage.

[0074]

The primers for use in such PCR methods can be judiciously established according to the sequence information on the gene of the invention which has been uncovered in accordance with the present invention and can be synthesized by the conventional procedure. Isolation and purification of the amplified DNA or RNA fragment can be carried out by the conventional techniques as mentioned hereinbefore, for example by gel electrophoresis.

[0075]

The nucleotide sequence of the gene of the present invention or any of the various DNA fragments which can be obtained as above can be determined in the routine manner, for example by the dideoxy method [Proc. Natl. Acad. Sci., USA., 74: 5463 (1977)], the Maxam-Gilbert method [Methods in Enzymology, 65: 499 (1980)] or, more expediently, by means of a commercial sequencing kit.

[0076]

With the gene of the present invention, for

example by utilizing a partial or full-length nucleotide sequence of this gene, the expression or non-expression of the gene of the present invention in an individual body or various tissues thereof can be specifically detected.

5 [0077]

This detection can be made in the routine manner. For example, determination at the cellular level by RNA amplification by RT-PCR [reverse transcribed-polymerase chain reaction; E. S. Kawasaki, et al., Amplification of
10 RNA In PCR Protocol, A Guide to methods and applications, Academic Press, Inc., San Diego, 21-27 (1991)], Northern blotting analysis [Molecular Cloning, Cold Spring Harbor Lab. (1989)], in situ RT-PCR [Nucl. Acids Res., 21, 3159-3166 (1993)] or in situ hybridization, the NASBA method
15 [nucleic acid sequence-based amplification, Nature, 350, 91-92 (1991)] and other techniques can be mentioned. The preferred is the RT-PCR-SSCP method.

[0078]

The primers for use in the PCR procedure are not
20 particularly restricted inasmuch as only the gene of the present invention can be specifically amplified, and can be judiciously established on the basis of the sequence information on the gene of the invention. Usually, for example, primers each having a partial sequence of the
25 gene of the invention and a length ranging from about 10

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to 35 nucleotides, preferably about 15-30 nucleotides, can be employed.

[0079]

Thus, the gene of the present invention includes
5 DNA fragments which can be used as specific primers and/or specific probes for detection of the human p51 gene of the invention.

[0080]

Such a DNA fragment can be defined as the DNA
10 characterized by its capability to hybridize with a DNA having the nucleotide sequence (145-1487) in Figs. 2-8 under stringent conditions. The stringent conditions mentioned above may be the conventional conditions used for primers and probes and, for that matter, not
15 particularly restricted but the above-mentioned conditions, namely 0.1% SDS-containing 0.2 x SSC at 50°C or 0.1% SDS-containing 1 x SSC at 60°C, can for example be mentioned.

[0081]

With the human p51 gene of the present invention,
20 the protein comprising the corresponding gene product (p51 protein) can be produced easily, on a large scale, and with good reproducibility by utilizing the conventional genetic engineering technology.

[0082]

25 The present invention, therefore, provides a

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protein including the p51 protein encoded by the gene of the invention, a vector harboring the gene of the invention, host cells transformed with the vector, and a method of producing the protein of the invention which
5 comprises growing the host cells.

[0083]

As a specific example of the protein of the present invention, there can be mentioned the p51 protein having the amino acid sequence shown in Fig. 1. It should,
10 however, be understood that the protein of the present invention is not limited to the p51 protein but includes its homologs. The homolog in this context includes the protein having an amino acid sequence derived from the above-mentioned amino acid sequence by deletion,
15 substitution or addition of one or a few or a plurality of amino acids and having said p51 activity. More particularly, the gene products of said p51 gene homologs (p51-related genes including alleles) can be mentioned.

[0084]

20 The protein of the present invention can be prepared by the conventional recombinant DNA technology [e.g. Science, 224, 1431 (1984); Biochem. Biophys. Res. Comm., 130, 692 (1985); Proc. Natl. Acad. Sci., USA., 80, 5990 (1983), etc.], based on the human p51 gene sequence
25 information provided by the present invention.

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[0085]

More particularly, the production of said protein is carried out in accordance with the procedure which comprises constructing a recombinant vector (expression vector) in which the gene coding for the desired protein may be expressed, transforming host cells with the resulting construct, culturing the transformant thus obtained, and harvesting the desired protein from the culture obtained.

10 [0086]

As said host cells, whichever of eucaryotic cells and procaryotic cells can be employed.

[0087]

The eucaryotic host cells include cells of vertebrae and yeasts, among others. As the former cells, the monkey cell line COS [Cell, 23: 175 (1981)], Chinese hamster ovarian cells and the dihydrofolate reductase-defective line thereof [Proc. Natl. Acad. Sci., USA., 77: 4216 (1980)] are often used but these are not exclusive choices. As to the latter cells, cells of yeasts are often used. Particularly, yeasts belonging to the genus Saccharomyces are usable with advantage.

20

[0088]

As the procaryotic host, Escherichia coli and Bacillus subtilis are often used. Particularly,

25

Escherichia coli K12 and the like are often used.

[0089]

The expression vector is not particularly restricted inasmuch as it harbors the gene of the present invention and permits expression of said gene, but is generally selected with reference to the kinds of host cells.

[0090]

When cells of a vertebrate are used as host cells, generally an expression vector having a promoter region upstream of the gene of the invention to be expressed, RNA splicing site, polyadenylation site and transcription termination sequence can be used and, where necessary, it may further include a replication origin. As an example of such expression vector, pSV2dhfr [Mol. Cell. Biol., 1, 854 (1981)] having the early promoter of SV40 can be mentioned.

[0091]

When cells of eucaryotic microorganisms such as yeasts are used as host cells, the expression vector which can be used includes pAM82 having the promoter of the acid phosphatase gene [Proc. Natl. Acad. Sci., USA., 80, 1 (1983)], and the vector for use in the present invention can be prepared by inserting the gene of the invention upstream of this promoter. Preferably, a fusion vector

obtainable by hybridization with a procaryotic gene can be used and, as specific examples of such vector, pGEX-2TK and pGEX-4T-2 each having a GST domain with a molecular weight of 26000 (derived from *S. japonicum*) can be mentioned.

[0092]

When procaryotic cells are used as host cells, the expression vector may for example be a plasmid vector which is replicable in the host cells and equipped with a promoter region and SD (Shine-Dalgarno) nucleotide sequence upstream of the gene so that the gene may be expressed therein and further an initiation codon (e.g. ATG) necessary for the initiation of protein synthesis. Particularly when cells of *Escherichia coli* (e.g. *Escherichia coli* K12) are used as host cells, generally pBR322 as such or modified is often used as the vector. However, these are not exclusive choices but other known bacterial strains and known vectors can also be employed. As the promoter, tryptophan (*trp*) promoter, *lpp* promoter, *lac* promoter, PL/PR promoter, etc. can be employed.

[0093]

The method of introducing the expression vector of the invention into the host cell and the transformation method is not particularly restricted, either, but various standardized methods can be utilized.

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[0094]

Culture of the resultant transformant can also be performed in the routine manner. By such culture, the object protein encoded by the gene of the invention is
5 expressed, produced, and accumulated in the transformant cell or secreted extracellularly or on the cell membrane.

[0095]

The medium for said culture can be judiciously selected from among the conventional culture media
10 according to the type of host cells adopted, and culture can also be carried out under conditions suited for growth of the host cells.

[0096]

The recombinant protein thus produced can be
15 optionally isolated and purified by various isolation procedures utilizing its physical, chemical or other properties [Seikagaku (Biochemical) Data Book II, pp.1175-1259, 1st Ed., 1st Impression, June 23, 1980, Tokyo Kagaku Dojin; Biochemistry, 25(25): 8274 (1986); Eur. J. Biochem.,
20 163: 313 (1987); etc.].

[0097]

The procedures mentioned above specifically include the standard reconstitution treatment, treatment with a protein precipitating agent (salting out),
25 centrifugation, osmotic shock method, sonic disruption,

ultrafiltration, various kinds of chromatography, e.g. molecular sieves chromatography (gel filtration), adsorption chromatography, ion exchange chromatography, affinity chromatography, high performance liquid chromatography (HPLC), etc., dialysis, and their combinations. The particularly preferred procedure is affinity chromatography using a column conjugated with a specific antibody against the protein of the invention.

[0098]

10 In the designing of the desired gene coding for the protein of the invention, the nucleotide sequence of the human p51 gene as identified by the nucleotide sequence (145-1487) in Figs. 2-8 can be utilized with advantage. If desired, this gene may be used with the
15 codons designating respective amino acid residues judiciously changed.

[0099]

Furthermore, the partial modification of the amino acid sequence encoded by the human p51 gene by the
20 substitution, deletion or addition of some of the amino acid residues or a partial amino acid sequence can be achieved by the various techniques mentioned hereinbefore, for example by site-specific mutagenesis.

[0100]

25 The protein of the present invention can be

synthesized by the standard technology for chemical synthesis in accordance with the amino acid sequence shown in Fig. 1. This technology includes the liquid-phase and solid-phase methods for peptide synthesis.

5 [0101]

More particularly, the synthetic technology includes the so-called stepwise elongation method in which one amino acid after another is sequentially coupled together in accordance with the amino acid sequence information and the fragment condensation method in which fragments each consisting of several amino acids are synthesized in advance and then coupled together. The polypeptide of the present invention can be synthesized by whichever of the above alternative methods.

15 [0102]

The condensation method for use in the above peptide synthesis may also be the conventional one, which includes but is not limited to the azide method, mixed acid anhydride method, DCC method, activated ester method, redox method, DPPA (diphenylphosphoryl azide) method, DCC + additive (1-hydroxybenzotriazole, N-hydroxysuccinamide, N-hydroxy-5-norbornene-2,3-dicarboximide or the like) method, and Woodward's method.

[0103]

25 The solvent for use in these methods can also be

judiciously selected from among the common solvents which are well known to those skilled in the art of peptide condensation. Examples of solvents include

dimethylformamide (DMF), dimethyl sulfoxide (DMSO),

- 5 hexaphosphoramidate, dioxane, tetrahydrofuran (THF), ethyl acetate and the like and mixed solvents thereof.

[0104]

The carboxyl groups of amino acids or peptides which are not to be involved in the reaction for said

- 10 peptide synthesis can be protected generally by esterification, for example in the form of a lower alkyl ester, e.g. methyl ester, ethyl ester, tert-butyl ester or the like, or an aralkyl ester, e.g. benzyl ester, p-methoxybenzyl ester, p-nitrobenzyl ester or the like.

- 15 [0105]

The amino acid having a functional group in its side chain, for example the hydroxyl group of the tyrosine residue, may be protected with an acetyl, benzyl,

benzyloxycarbonyl, tert-butyl or other group, although

- 20 this protection is not indispensable. Furthermore, the guanidino group of an arginine residue, for instance, can be protected with a suitable protective group such as nitro, tosyl, p-methoxybenzenesulfonyl, methylene-2-sulfonyl, benzyloxycarbonyl, isobornyloxycarbonyl,

- 25 adamantyloxycarbonyl or the like.

[0106]

The deprotection reactions of such protected amino acids, peptides and end product protein of the present invention for removal of the protective groups can also be carried out by the conventional method, for example the catalytic reduction method or the method using liquid ammonia/sodium, hydrogen fluoride, hydrogen bromide, hydrogen chloride, trifluoroacetic acid, acetic acid, formic acid, methanesulfonic acid or the like.

10 [0107]

The protein thus produced can be purified by the procedure which is conventionally utilized in the field of peptide chemistry, such as said various methods, such as ion exchange resin, partition chromatography, gel permeation chromatography, countercurrent distribution, etc.

[0108]

The protein of the present invention can be used with advantage as an immunogen in the preparation of a specific antibody to the p51 protein (included in polypeptide of the invention), and by using such an immunogen, the desired antiserum (polyclonal antibody) and monoclonal antibody can be acquired.

[0109]

25 The antibody production technology as such is

well understood by those skilled in the art and, in the practice of the present invention, too, the conventional methods can be utilized [e.g. Zoku Seikagaku Jikken Koza (Supplemental Biochemical Experimental Series), Methods
5 for Immunobiochemical Research, edited by Japanese Biochemical Society (1986)]. The antibody thus obtained can be used with advantage, for example in the purification of the p51 protein and the immunological assay or characterization of the protein.

10 [0110]

Furthermore, the protein of the present invention finds application in the pharmaceutical field, in the manufacture of pharmaceutical products containing the protein as an active ingredient.

15 [0111]

The present invention, therefore, is further directed to said pharmaceutical products comprising the protein of the invention.

[0112]

20 The protein mentioned above includes its pharmaceutically acceptable salt. Such salt includes the following salts produced by known methods in the art: nontoxic alkali metal, alkaline earth metal and ammonium salts, such as sodium, potassium, lithium, calcium,
25 magnesium, barium and ammonium salts. Furthermore, said

salt includes nontoxic acid addition salts obtainable by reacting the peptide of the invention with a suitable organic or inorganic acid. The representative nontoxic acid addition salts are the hydrochloride, hydrobromide, sulfate, bisulfate, acetate, oxalate, valerate, oleate, 5 laurate, borate, benzoate, lactate, phosphate, p-toluenesulfonate (tosylate), citrate, maleate, fumarate, succinate, tartrate, sulfonate, glycolate, ascorbate, benzenesulfonate and naphthalate, among others.

10 [0113]

The present invention further comprises a pharmaceutical composition or dosage form which contains a pharmacologically effective amount of the protein of the invention as an active ingredient together with a suitable 15 nontoxic pharmaceutical carrier or diluent.

[0114]

The pharmaceutical carrier which can be utilized in said pharmaceutical composition (or dosage form) includes the diluents and excipients which are commonly 20 used according to the mode of use of the pharmaceutical preparation, such as filler, volume builder, binder, humectant, disintegrator, surfactant, lubricant, etc., and these are selectively employed according to the unit dosage form of the pharmaceutical preparation.

25 [0115]

The particularly preferred pharmaceutical preparation of the present invention is produced by using various formulating substances which can be incorporated in the conventional protein preparation, such as the
5 stabilizer, bactericide, buffer, isotonizing agent, chelating agent, pH control agent, surfactant, etc., in suitable proportions.

[0116]

The stabilizer mentioned above includes but is
10 not limited to human serum albumin, ordinary L-amino acids, saccharides and cellulose derivatives and these can be used independently or in combination with a surfactant or the like. Particularly in the combination use, the stability of the active ingredient can be further improved
15 in certain cases.

[0117]

The L-amino acids mentioned above are not particularly restricted but may be glycine, cysteine, glutamic acid and so on.

20 [0118]

The saccharides mentioned above include monosaccharides such as glucose, mannose, galactose, fructose, etc., sugar alcohols such as mannitol, inositol, xylitol, etc., disaccharides such as sucrose, maltose,
25 lactose, etc., polysaccharides such as dextran,

hydroxypropylstarch, chondroitin sulfate, hyaluronic acid, etc., and their derivatives.

[0119]

The surfactant is not particularly restricted,
5 either. Thus, ionic surfactants and nonionic surfactants, such as surfactants in the polyoxyethylene glycol sorbitan alkyl ester, polyoxyethylene alkyl ether, sorbitan monoacyl ester and fatty acid glyceride series can be mentioned.

10 [0120]

The cellulose derivatives are not particularly restricted, either, but methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropylmethylcellulose, carboxymethylcellulose
15 sodium, etc. can be mentioned.

[0121]

The suitable level of addition of the saccharide per μg of the active ingredient is not less than about 0.0001 mg, preferably about 0.01-10 mg. The level of
20 addition of the surfactant per μg of the active ingredient may suitably be not less than about 0.00001 mg, preferably about 0.0001-0.01 mg. The level of addition of human serum albumin per μg of the active ingredient may suitably be not less than about 0.0001 mg, preferably
25 somewhere within the range of about 0.001-0.1 mg. The

level of addition of said amino acid per μ g of the active ingredient is suitably about 0.001-10 mg. The level of addition of the cellulose derivative per μ g of active ingredient is suitably not less than about 0.00001 mg,
5 preferably about 0.001-0.1 mg.

[0122]

The amount of the active ingredient in the pharmaceutical dosage form can be liberally selected from a broad range but can be judiciously selected from the
10 range of generally about 0.00001-70 weight %, preferably about 0.0001-5 weight %.

[0123]

The pharmaceutical dosage form of the present invention may be supplemented with various additives, such
15 as a buffer, an isotonizing agent, a chelating agent, etc. The buffer mentioned above includes boric acid, phosphoric acid, acetic acid, citric acid, ϵ -aminocaproic acid, glutamic acid, etc. and/or the corresponding salts (alkali metal or alkaline earth metal salts, e.g. sodium salt,
20 potassium salt, calcium salt, magnesium salt, etc.). The isotonizing agent includes but is not limited to sodium chloride, potassium chloride, sugars and glycerin. The chelating agent includes sodium edetate and citric acid, among others.

25

[0124]

The pharmaceutical composition of the present invention can be used not only as it is in the form of a solution but also provided in the form of a lyophilized product which can be preserved and extemporaneously
5 reconstituted with water or a buffer solution inclusive of physiological saline to a suitable concentration.

[0125]

The unit dosage form for the pharmaceutical composition of the present invention can be selected from
10 various alternatives according to the therapeutic purpose, and includes solid dosage forms, such as tablets, pills, powders, fine powders, granules, capsules, etc. and liquid dosage forms, such as solutions, suspensions, emulsions, syrups and elixirs. These dosage forms can be further
15 classified as the oral, parenteral, transnasal, vaginal, rectal (suppository) and sublingual dosage forms, ointments and other products, and each product can be manufactured in accordance with the established formulation and molding/processing procedure.

20 [0126]

Taking the manufacture of tablets as an example, the pharmaceutical carrier which can be used includes various excipients such as lactose, sucrose, sodium chloride, glucose, urea, starch, calcium carbonate, kaolin,
25 crystalline cellulose, silicic acid, potassium phosphate,

etc.; binders such as water, ethanol, propanol, simple syrup, glucose solution, starch solution, gelatin solution, carboxymethylcellulose, hydroxypropylcellulose, methylcellulose, polyvinylpyrrolidone, etc. disintegrators
5 such as carboxymethylcellulose sodium, carboxymethylcellulose calcium, low-substitution-degree hydroxypropylcellulose, dry starch, sodium alginate, agar powder, laminaran powder, sodium hydrogencarbonate, calcium carbonate, etc.; surfactants such as
10 polyoxyethylene sorbitan fatty acid esters, sodium lauryl sulfate, stearyl monoglyceride, etc.; disintegration inhibitors such as sucrose, stearin, cacao butter, hydrogenated oil, etc.; absorption promoters such as quaternary ammonium bases, sodium lauryl sulfate, etc.;
15 humectants such as glycerin, starch, etc.; adsorbents such as starch, lactose, kaolin, bentonite, colloidal silica, etc.; and lubricants such as purified talc, stearate salts, boric acid powder, polyethylene glycol and so on.

[0127]

20 Furthermore, tablets may optionally be coated with a usual coating material to provide sugar-coated tablets, gelatin-coated tablets, enteric-coated tablets, film-coated tablets, etc. or even processed into multilayer tablets such as double-layer tablets.

25 [0128]

Pills can be manufactured by using various pharmaceutical carriers inclusive of excipients such as glucose, lactose, starch, cacao butter, hydrogenated vegetable oil, kaolin, talc, etc.; binders such as gum Arabic, tragacanth powder, gelatin, ethanol, etc.; and disintegrators such as laminaran, agar and so on.

[0129]

Capsules can be prepared by blending the active ingredient of the present invention with said various pharmaceutical carriers and filling capsule shells, such as hard gelatin capsule shells or soft capsule shells, with the resulting composition.

[0130]

Liquid dosage forms for oral administration include pharmaceutically acceptable solutions, emulsions, suspensions, syrups, elixirs, etc. as prepared by using conventional inert diluents, such as water, and may further contain auxiliary agents such as wetting agents, emulsifiers and suspending agents. These dosage forms can be manufactured by the conventional procedure.

[0131]

Liquid dosage forms for parenteral or nonoral administration, such as sterile aqueous or nonaqueous solutions, emulsions and suspensions, can be manufactured using a diluent such as water, ethyl alcohol, propylene

glycol, polyethylene glycol, ethoxylated isostearyl alcohol, polyoxylated isostearyl alcohol, polyoxyethylene sorbitan fatty acid ester, a vegetable oil, e.g. olive oil, and may be formulated with an injectable organic ester, such as ethyl oleate. Furthermore, such preparations may be supplemented with conventional solubilizers, buffers, wetting agents, emulsifiers, suspending agents, preservatives, dispersants and other additives. Sterilization may be carried out by filtration through a bacterial filter, formulation of a bactericide, irradiation, heating or the like. Furthermore, said preparations can be processed into sterile solid dosage forms which can be extemporaneously dissolved in sterile water or a suitable sterilizable medium.

15 [0132]

In the manufacture of rectal suppositories or a dosage form for vaginal administration, there can be employed a pharmaceutical carrier such as polyethylene glycol, cacao butter, a higher alcohol, a higher alcohol ester, gelatin, semisynthetic glyceride or the like.

[0133]

In the manufacture of ointments inclusive of pastes, creams and gels, there can be employed such diluents as white petrolatum, paraffin, glycerin, cellulose derivatives, propylene glycol, polyethylene

glycol, silicone oil, bentonite and vegetable oils such as olive oil.

[0134]

Compositions for transnasal or sublingual
5 administration can be prepared with the well-known
standard excipient in the conventional manner.

[0135]

Where necessary, coloring agents, preservatives,
flavoring agents, corrigents, sweeteners, and other
10 medicinal substances can be incorporated in the
pharmaceutical products of the present invention.

[0136]

The method of administering said pharmaceutical
product is not particularly restricted but should be
15 judiciously selected according to the dosage form, patient
factors such as age and sex, severity of illness and other
factors. For example, said tablets, pills, solutions,
suspensions, emulsions, granules and capsules are
administered orally. The parenteral product is used alone
20 or mixed with a conventional infusion, such as a glucose
or amino acid infusion, and administered intravenously or,
where necessary, administered alone intramuscularly,
intradermally, subcutaneously or intraperitoneally. The
suppositories are administered rectally; the vaginal
25 preparation is administered into the vagina. The

transnasal preparation is administered into the nostrils; sublingual tablets are administered buccally; and ointments are topically administered transdermally.

[0137]

5 The amount of the active ingredient of the invention in the pharmaceutical product and the dosage thereof are not particularly restricted but can be judiciously selected from a broad range according to the expected therapeutic effect, administration method,
10 treatment course or duration, patient factors such as age and sex, and other factors. Generally, the dosage is usually about 0.01 μ g ~ 10 mg/kg body weight/day, preferably about 0.1 μ g ~ 1 mg/kg b.d./day, and this amount can be administered once or in a few divided doses
15 daily.

[0138]

 The present invention further provides a method of gene therapy utilizing the human p51 gene of the invention. This therapeutic method may be regarded as a
20 method for imparting the wild type p51 gene functions to the cells harboring a mutant p51 gene. By imparting the normal functions inherently possessed by the wild type p51 gene to cells, neoplastic growth of the recipient/target cells can be inhibited. The wild type p51 gene mentioned
25 above can be transferred into the objective cells by means

25 The vectors which can be used in the transfer of

the object gene for both such recombination and extrachromosomal maintenance of the gene are already known in the art and any of the known vectors can be used in the practice of the present invention. For example, a virus
5 vector or plasmid vector which harbors a copy of p51 gene linked to an expression control element and is capable of insuring expression of the gene product within the target cells can be mentioned. As such a vector, the expression vectors mentioned above can be generally used but
10 preferably vectors constructed using such source vectors as the vectors disclosed in U.S. Patent No. 5252479 and PCT WO 93/07282 (pWP-7A, pWP-19, pWU-1, pWP-8A, pWP-21 and/or pRSVL, among others) or pRC/CMV (Invitrogen). The more preferred are the various virus vectors described
15 hereinafter.

[0142]

As the promoter for the vector to be used in gene therapy, the promoters intrinsic to the target affected tissues in various diseases can be utilized with
20 advantage.

[0143]

Specific examples may be cited below. For the liver, for instance, albumin, α -fetoprotein, α 1-antitrypsin, transferrin, transthyretin, etc. can be
25 mentioned by way of example. For the colon, carboxyl

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anhydrase I, carcinoembryon antigen, etc. can be mentioned. For the uterus and placenta, estrogen, aromatase cytochrome P450, cholesterol side-chain cleaving P450, 17 α -hydroxylase P450, etc. can be mentioned.

5 [0144]

For the prostate, prostatic antigen, gp91-fox gene, prostate-specific kallikrein, etc. can be mentioned. For the mamma, erb-B2, erb-B3, β -casein, β -lactoglobulin, whey protein, etc. can be mentioned. For the lung, the
10 activator protein C uroglobulin, among others, can be mentioned. For the skin, K-14-keratin, human keratin 1 or 6, leucrin, etc. can be mentioned.

[0145]

For the brain, neuroglia fiber acid protein,
15 mature astrocyte-specific protein, myelin, tyrosine hydroxylase pancreatic villin, glucagon, Langerhans islet amyloid polypeptide, etc. can be mentioned. For the thyroid, thyroglobin, calcitonin, etc. can be mentioned. For the bone, α 1 collagen, osteocalcin, bone
20 sialoglycoprotein, etc. can be mentioned. For the kidney, renin, liver/bone/kidney alkaline phosphatase, erythropoietin, etc. can be mentioned. For the pancreas, amylase, PAP1, etc. can be mentioned.

[0146]

25 The gene (the whole or a fragment) to be used

for the construction of a gene transfer vector can be easily produced and acquired by the standard genetic engineering technology based on the nucleotide sequence information about the p51 gene of the invention as

5 mentioned hereinbefore.

[0147]

The introduction of such a gene transfer vector into cells can be carried out by various alternative techniques known to those skilled in the art, such as

10 electroporation, calcium phosphate coprecipitation, viral transduction and so on. The cells transformed with the wild type p51 gene can be used as they are in the isolated form as the agent for tumor suppression or inhibition of cancer metastasis or as a model system for therapeutics

15 research.

[0148]

In gene therapy, said gene transfer vector can be introduced into the tumor cells of a patient by topical administration to the tumor site or by systemic

20 administration to the patient by injection. By systemic administration, the gene can be caused to arrive at any tumor cells metastable to other sites. If the transformed cells cannot be permanently taken up in the chromosomes of the target tumor cells, the above administration may be

25 repeated periodically.

[0149]

The method of gene therapy according to the present invention includes both the in vivo method which comprises administering a material for said gene transfer
5 (gene transfer vector) directly into the body and the ex vivo method which comprises withdrawing the target cells from the patient's body, introducing the gene extracorporeally, and returning the cells into the body.

[0150]

10 A further possible alternative is a gene therapy using a ribozyme which comprises introducing the human p51 gene directly into cells and cleaving the RNA chain with the ribozyme which is an active molecule.

[0151]

15 The gene transfer vector harboring the human p51 gene of the invention or a fragment thereof and a gene-therapeutic agent comprising cells transformed with the human p51 gene by means of said vector as an active ingredient are directed especially to the therapy of
20 cancers but the gene therapy (treatment) mentioned above can be applied also to the therapy of hereditary diseases and viral diseases such as AIDS, as well as for the purpose of gene labeling.

[0152]

25 The target cells to which the gene is

transferred can be judiciously selected according to the object of gene therapy (treatment). For example, as the target cells, not only cancer cells and tumor tissues but also lymphocytes, fibroblasts, hepatocytes, hematopoietic stem cells and other cells can be mentioned.

[0153]

The method of introducing the gene into cells in the gene therapy includes viral transfer methods and non-viral transfer methods.

[0154]

As the viral transfer method, the method using a retrovirus vector, for instance, can be used in view of the fact that the human p51 gene is a foreign gene which is expressed in normal cells. As other virus vectors, adenovirus vector, HIV (human immunodeficiency virus) vector, adeno-associated virus (AAV) vector, herpes virus vector, herpes simplex virus (HSV) vector and Epstein-Barr virus (EBV) vector, etc. can be mentioned.

[0155]

The non-viral gene transfer method includes the calcium phosphate coprecipitation method; the membrane-fusion liposome method which comprises fusing DNA-containing liposomes with an inactivated Sendai virus as exposed to UV radiation for gene destruction to construct membrane-fusion liposomes and introducing the DNA into

The ligand-DNA complex method mentioned above includes the method which comprises using the asialoglycoprotein receptor expressed in liver cells as the target and an asialoglycoprotein as the ligand [Wu, et al., J. Biol. Chem., 266, 14338 (1991); Ferkol, et al.,

FASEB J., 7, 1081-1091 (1993)] and the method which comprises using the transferrin receptor expressed at a high level in tumor cells as the target and transferrin as the ligand [Wagner et al., Proc. Natl. Acad. Sci., USA.,
5 87, 3410 (1990)], among others.

[0157]

Furthermore, the gene transfer method which can be used may be a suitable combination of said biological and physical gene transfer methods. As such a combination
10 method, there can be mentioned the method in which a plasmid DNA having a given size is used in combination with a polylysine-conjugated antibody specific to adenovirus hexon protein. According to this method, the complex formed is bound to the adenovirus vector and the
15 resulting trimolecular complex can be used to infect cells and thereby transfer the gene of the present invention. In accordance with this method, effective binding, endogenization and endosome degradation can take place before the DNA coupled to the adenovirus vector is damaged.
20 Moreover, said liposome/DNA complex may mediate the gene transfer in vivo.

[0158]

The method of constructing a virus vector for transfer of the gene of the invention and the method of
25 introducing the gene into the target cells or target

tissue are now described.

[0159]

The retrovirus vector system comprises the virus
vector and helper cells (packaging cells). The helper
5 cells mentioned above are cells in which genes such as
retrovirus structural protein gag (the structural protein
in the virus particle), pol (reverse transcriptase) and
env (shell protein) have been expressed but have not
formed virus particles. On the other hand, the virus
10 vector has a packaging signal and LTR (long terminal
repeats) but are devoid of structural genes necessary for
virus replication, such as gag, pol and env. The
packaging signal is a sequence serving as the tag in the
assembling of a virus particle and the selective genes
15 (neo, hyg) and the desired gene (p51 gene or a fragment
thereof) incorporated in the cloning site are inserted in
place of the virus gene. Here, in order to acquire a high
titer of virus particles, it is important to make the
insert as short as possible, broaden the packaging signal
20 by including a part of the gag gene and use care not to
leave the ATG of the gag gene.

[0160]

By introducing the vector DNA harboring the
object p51 gene into the helper cells, the vector genome
25 RNA is packaged with the virus structural protein of the

helper cells to form and secrete virus particles. After the virus particle as the recombinant virus has infected the target cell, the DNA reverse-transcribed from the virus genome RNA is integrated into the cell nucleus and
5 the gene inserted into the vector is expressed.

[0161]

As the method of improving the efficiency of transfer of the object gene, the method using a fragment containing the cell adhesion domain of fibronectin, the
10 heparin-binding site and conjugation segment can be employed [Hananberg, H., et al., Exp. hemat., 23, 747 (1995)].

[0162]

As an example of the vector for use in the above
15 retrovirus vector system, the retrovirus derived from mouse leukemia virus [McLachlin, J. R., et al., Proc. Natl. Acad. Res. Molec. Biol., 38, 91-135 (1990)] can be mentioned.

[0163]

20 The method comprising using the adenovirus vector is now described in detail. Construction of said adenovirus vector can be carried out in accordance with the methods of Berkner [Berkner, K. L., Curr. Topics Microbiol. Immunol., 158, 39-66 (1992)], Yasuhiro
25 Setoguchi, et al. [Setoguchi, Y., et al., Blood, 84, 2946-

2953 (1994)], Hiromi Kanegae, et al. [Experimental Medicine, 12, 28-34 (1994)], and Ketner et al. [Ketner, G., et al., Proc. Natl. Acad. Sci., USA., 91, 6186-6190 (1994)].

5 [0164]

For example, for the construction of a nonproliferative adenovirus vector, the early gene E1 and/or E3 gene regions of adenovirus are first removed. Then, a plasmid vector harboring the object foreign gene expression unit (consisting of the gene to be transferred, which is the p51 gene in the present invention, a promoter for transcription of the gene, and poly-A which imparts stability to the transcript) and a portion of the adenovirus genome DNA and a plasmid harboring the adenovirus genome are used to concurrently transfect cells, e.g. 293-cells. By causing a homologous recombination to take place between the two and thereby substitute the gene expression unit for E1, the nonproliferative adenovirus vector harboring the p51 gene according to the present invention can be constructed. It is also possible to integrate the adenovirus genome DNA into the cosmid vector to construct a 3'-end adenovirus vector with the terminal protein added. Furthermore, the YAC vector can also be utilized in the construction of a recombinant adenovirus vector.

10
15
20
25

-64-

[0165]

The production of an adeno-associated virus (AAV) vector is now briefly described. AAV was discovered as a small-sized virus contaminating a culture system of
5 adenovirus. Of this virus, the parvovirus genus which does not require a helper virus for replication but proliferates autonomously in the host cell and the dependvirus which requires a helper virus have been confirmed. This AAV is one of the common viruses which
10 has a broad host range and infects a variety of cells. Its genome is a linear single-stranded DNA consisting of 4680 nucleotides and the 145 nucleotides at either terminus have a characteristic sequence called ITR (inverted terminal repeat). This ITR region is a
15 replication initiation point and plays the role of a primer. Furthermore, this ITR is indispensable to the packaging to the virus particle and the integration into the chromosomal DNA of the host cell. In addition, with regard to the virus protein, the left-half of the genome
20 codes for a nonstructural protein, that is the regulatory protein Rep which controls replication and transcription.

[0166]

Construction of a recombinant AAV can be carried out by utilizing the property of AAV to be integrated with
25 chromosomal DNA, whereby a vector for transfer of the

desired gene can be constructed. More particularly, in accordance with this method, a plasmid (AAV vector plasmid) harboring the object gene to be transferred (human p51 gene) inserted between the remnant ITRs at both the 5'- and 3'-ends of the wild type AAV is first constructed. On the other hand, the virus protein necessary for virus replication and construction of the virus particle is supplied from an independent helper plasmid. It is insured that there will be no nucleotide sequence common to both plasmids so that a recombinant wild-type virus will not emerge. Then, both plasmids are introduced by transfection into, for example, 293-cells, which are further infected with adenovirus (which may be nonproliferative type when 293-cells are used) as the helper virus, whereby the objective recombinant AAV of the nonproliferative type is produced. Since this recombinant AAV exists in the nucleus, it is recovered by freeze-thaw and the contaminant adenovirus is inactivated by heating at 56°C. Where necessary, the recombinant AAV is isolated and concentrated by ultracentrifugation with cesium chloride. In this manner, the objective recombinant AAV for transfer of the object gene can be acquired.

[0167]

Construction of the HIV vector can be carried out typically in accordance with the method of Shimada et

al. [Shimada, T., et al., J. Clin. Invest., 88, 1043-1047 (1991)].

[0168]

Since the HIV virus specifically infects helper
5 T cells with CD4 as the receptor, a tissue-specific gene transfer HIV vector adapted for specific introduction of a gene into human CD4-positive cells can be constructed. This HIV vector is optimal for the gene therapy of AIDS.

[0169]

10 Construction of a recombinant HIV vector can be carried out typically as follows. First, the packaging plasmid CGPE is constructed in such a manner that the structural genes gag, pol and env and the control genes (tat, rev, etc.) necessary for expression thereof may be
15 expressed with the cytomegalovirus (CMV) promoter and the human globin gene poly A signal (poly A). Then, the vector plasmid HXN can be constructed so as to permit efficient proliferation in COS cells by inserting the bacterial neomycin-resistant gene (neoR) having a promoter
20 for thymidine kinase (TK) as a marker gene between the two LTRs of HIV and further inserting a SV40 replication mechanism into the basal plasmid vector. As the above packaging plasmid CGPE and vector plasmid HXN are concurrently introduced by transfection into COS cells,
25 the objective neoR gene-integrated recombinant virus is

produced and released into the culture medium in a large quantity.

[0170]

Production of the EBV vector can be carried out typically in accordance with the method of Shimidzu et al
5 [Norio Shimidzu, Jinzo Takada, Cell Engineering, 14(3), 280-287 (1995)].

[0171]

The production of an EBV vector for transfer of
10 the gene of the present invention is now briefly described. EB virus (Epstein-Barr virus: EBV) is a virus belonging to the herpes family and was isolated from cultured cells derived from Burkitt lymphoma by Epstein and coworkers in 1964 [Kieff, E. and Liebowitz, D.: Virology, 2nd ed. Raven
15 Press, New York, 1990, pp.1889-1920]. The EBV has cell-transforming activity and, therefore, in order that it may be utilized as a gene transfer vector, the virus defective of this tranforming activity must be prepared. This can be done as follows.

20

[0172]

Thus, in the first place, an EBV genome close to the target DNA with which the desired foreign gene is to be integrated is cloned. With this clone, a foreign gene DNA fragment and a drug-resistant gene are integrated to
25 prepare a vector for production of a recombinant virus.

Then, the vector for construction of a recombinant virus is excised with suitable restriction enzymes and introduced by transfection into EBV-positive Akata cells. The recombinant virus produced by the homologous recombination can be recovered, together with the wild type Akata EBV, through virus production stimulation by anti-surface immunoglobulin treatment. This is used to infect EBV-negative Akata cells and a resistant strain is selected in the presence of the drug to obtain the desired Akata cells infected exclusively by the recombinant virus and free from the wild type EBV. Then, by inducing virus activity in the recombinant virus-infected Akata cells, the objective recombinant virus vector can be produced in a large quantity.

15 [0173]

Production of a non-virus vector for the introduction of the desired gene into target cells without use of a recombinant virus vector can be carried out by the gene transfer technique using a membrane fusion liposome preparation. This is a technique such that by imparting fusion activity to a membrane liposome (a vesicle having a lipid bilayer structure), the contents of the liposome are directly introduced into the cell.

[0174]

25 Introduction of the gene by means of such

membrane fusion liposomes can be carried out typically in accordance with the method of Nakanishi et al. [Nakanishi, M., et al., Exp. Cell Res., 159, 399-499 (1985); Nakanishi, M., et al., Gene introduction into animal tissues. In Trends and Future Perspectives in Peptide and Protein Drug Delivery (ed. by Lee, V. H. et al.), Harwood Academic Publishers GmbH, Amsterdam, 1995, pp. 337-349].

[0175]

This method of gene transfer by means of said membrane fusion liposomes is briefly described below. Thus, liposomes in which Sendai virus with its gene inactivated by UV irradiation, the object gene and a high molecular substance, such as protein, have been entrapped is fused at 37°C. This membrane fusion liposome has a structure called "pseudovirus" which consists of a liposome-derived inner cavity and an outer spike structure similar to that of the virus envelope. After purification by sucrose density gradient centrifugation, the membrane fusion liposomes are caused to get adsorbed on the target cultured cells or tissue cells at 4°C. Then, as the temperature is increased to 37°C, the contents of the liposomes are introduced into the cell, whereby the desired gene can be transferred to the target cells. The lipid for said liposome in this case is a synthetic phospholipid composed of 50% (by mole) each of cholesterol

and lecithin and having a negative charge and is preferably formed as a unilamellar liposome with a diameter of 300 nm.

[0176]

5 As an alternative method of introducing the gene into the target cell by means of liposomes, the gene transfer method using cationic liposomes can be mentioned. This method can be practiced in accordance with the method of Yagi et al. [Yagi, K., et al., B. B. R. C., 196, 1042-
10 1048 (1993)]. Thus, with attention paid to the fact that plasmids and cells are both negatively charged, a positive charge is imparted to both the external and internal surfaces of the liposome membrane so that the uptake of the plasmid is increased by static electricity to enhance
15 the interaction with the cells. The liposome used in this case is preferably a multilamellar large vesicle (MLV) having a positive charge, although it is possible to use a large unilamellar vesicle (LUV) or a small unilamellar vesicle (SUV) to construct a complex with the plasmid for
20 introduction of the desired gene.

[0177]

The method of preparing a plasmid-containing cationic MLV is briefly described below. In the first place, a chloroform solution containing the lipid TMAG (N-
25 (α -trimethylammonioacetyl)-didodecyl-D-glutamate

chloride), DLPC (dilauroyl phosphatidylcholine) and DOPE (dioleoyl phosphatidylethanolamine) in a molar ratio of 1:2:2 is prepared (lipid concentration: 1 mM). Then, a total of 1 μ mol of lipid is placed in a centrifuge tube and the chloroform is distilled off under pressure using a rotary evaporator to prepare a lipid thin film. The residual chloroform is completely removed under reduced pressure and the film is dried. Then, 0.5 ml of Mg and Ca-containing Dulbecco's phosphate-buffered saline containing 20 μ g of the gene transfer plasmid is added and, after nitrogen purging, the mixture is stirred with a vortex mixer for 2 minutes to give a suspension of the gene-harboring plasmid-containing cationic MLV.

[0178]

The following is an example of use of the plasmid-containing cationic MLV as a gene therapy agent. For example, the expression plasmid integrated with the cDNA of the object gene is entrapped in an amount of 0.6 μ g as DNA per 30 nmole of liposome lipid in the above cationic MLV and the liposomes are suspended in 2 μ l of phosphate-buffered saline. This suspension is administered to the target cells extracted from the patient or the target patient tissue every other day.

[0179]

In this connection, in the guidelines

established by the Ministry of Health and Welfare of Japan,
the gene therapy is defined as "to administer a gene or a
gene-integrated cell into the human body for the therapy
of a disease". However, the gene therapy in the context
5 of the present invention encompasses not only the therapy
falling under the above definition but also the therapy of
various diseases inclusive of cancer which comprises
introducing a gene characterized as a tumor suppressor
gene, such as the human p51 gene, into said target cells
10 and the practice which comprises introducing a marker gene
or cells integrated with such a marker gene into the human
body.

[0180]

In the gene therapy according to the present
15 invention, the method of introducing the object gene into
the target cells or tissues includes the following two
representative methods.

[0181]

The first method comprises isolating the target
20 cells from the patient to be treated, growing the cells
extracorporeally, for example in the presence of
interleukin-2 (IL-2) or the like, introducing the p51 gene
ligated to the retrovirus vector into the cells, and
retransplanting the resulting cells (ex vivo method).
25 This method is suited for the therapy of ADA deficiency

syndrome, hereditary diseases and cancers associated with defective genes, AIDS, and other diseases.

[0182]

The second method is a direct gene transfer method (a direct method) which comprises injecting the object gene (human p51 gene) directly into the patient's body or target site, such as a tumor tissue (direct method).

[0183]

10 More particularly, the above first method of gene therapy can be carried out typically as follows. Thus, the mononuclear cells isolated from the patient are separated from monocytes with a blood separator, the harvested cells are cultured in the presence of IL-2 in a
15 suitable medium such as AIM-V medium for about 72 hours, and the vector harboring the gene to be introduced (human p51 gene) is added. For enhanced gene transfer efficiency, the system may be centrifuged at 2500 rpm in the presence of protamine at 32°C for 1 hour and incubated under 10%
20 carbon dioxide gas at 37°C for 24 hours. After the above procedure is repeated a few times, the cells are further cultured in the presence of IL-2 in AIM-V or other medium for 48 hours. The cells are washed with saline, viable cells are counted, and the gene transfer efficiency is
25 evaluated by carrying out said in situ PCR or, when the

object function is enzymatic activity, assaying the activity to confirm the gene transfer effect.

[0184]

In addition, a safety check comprising culture
5 of the bacteria and fungi contaminating the cultured cells
and testing for mycoplasma infection and for endotoxin is
carried out to confirm safety. Then, the cultured cells
integrated with a predicted effective dose of the gene
(human p51 gene) are returned to the patient by
10 intravenous drip injection. This procedure is repeated at
an interval of a few weeks or a few months for gene
therapy.

[0185]

The dosage of the virus vector can be
15 judiciously selected according to the kind of target cell.
Usually, in terms of virus titer, a dose within the range
of 1×10^3 cfu ~ 1×10^8 cfu is used per 1×10^8 target cells.

[0186]

An alternative version of the above first method,
20 which can be employed, comprises co-culturing virus-
producing cells containing a retrovirus vector harboring
the desired gene (human p51 gene) with, for example, the
patient's cells to introduce the gene (human p51 gene)
into the target cells.

25 [0187]

In carrying out the second method of gene therapy (direct method), a preliminary experiment, particularly an ex vivo experiment, is preferably performed to confirm whether the object gene (human p51
5 gene) may be actually transferred or not by a PCR of the vector gene cDNA or an in situ PCR assay or confirm the desired effect of therapy resulting from the transfer of the object gene (human p51 gene), for example an elevation in specific activity or an enhancement or suppression of
10 growth of target cells. Furthermore, when a virus vector is used, it is, of course, important, in conducting a gene therapy, to confirm the safety of introduction of the gene by carrying out the PCR to search for proliferative retrovirus, determining the reverse transcriptase activity,
15 or monitoring the membrane protein (env) gene by a PCR technique.

[0188]

When the method of gene therapy according to the present invention is applied to cancers or malignant
20 tumors in particular, a typical protocol for cancer therapy may comprise isolating cancer cells from the patient, treating the cells with an enzyme or the like to establish a cultured cell line, introducing the desired gene into the target cancer cells by means of retrovirus
25 or the like, carrying out a screening with G418 cells,

determining the amount of expression of IL-12 or the like (in vivo), subjecting the cells to radiation treatment, and inoculating the cells into the patient's tumor or paratumor.

5 [0189]

The herpes simplex virus-thymidine kinase (HSV-TK) gene reportedly causes cell death due to division aging, particularly by converting the nucleotide analog gancyclovir (GCV) to a toxic intermediate, and there is
10 known a gene therapy using this gene in tumors [U.S. Patent No. 5631236; JP Kohyo H9-504784]. This method is a method of gene therapy which utilizes the phenomenon that when cells capable of producing a retrovirus vector harboring said HSV-TK gene, known as a suicide gene, are
15 injected and, one week later, the antiviral agent GCV is administered, the GCV in the gene-transformed cells is activated by phosphorylation to induce death of these cells and death of the surrounding non-gene-transferred cells due to cell contact through the gap junction. The
20 gene transfer vector of the invention or cells containing this vector can be used in the above gene therapy as well.

[0190]

An alternative method of gene therapy comprises preparing immunoliposomes containing the gene (human p51
25 gene) coupled to the antibody capable of coupling to the

target cell surface to introduce the entrapped cDNA into the target cells selectively and with good efficiency. Feasible as well is a gene therapy which comprises administering said cytokine gene-harboring virus vector
5 and said suicide gene-harboring adenovirus at one and the same time. These methods invariably fall within the expertise of those skilled in the art.

[0191]

The present invention further provides a
10 pharmaceutical composition or agent (gene-therapeutic agent) comprising a pharmcologically effective amount of cells to which either the gene transfer vector or the object gene (e.g. human p51 gene or the like) of the invention has been transferred as an active ingredient
15 together with a suitable nontoxic pharmaceutical carrier or diluent.

[0192]

The pharmaceutical carrier which can be formulated in the pharmaceutical composition (preparation)
20 of the present invention includes the conventional diluents and excipients, such as filler, volume builder, binder, humectant, disintegrator, surfactant, lubricant, etc., which are commonly employed according to the method of use of the preparation and these carriers can be
25 selectively used with reference to the desired unit dosage

form.

[0193]

The unit dosage form for the pharmaceutical composition of the present invention includes the same dosage forms as those mentioned for the p51 protein and can be judiciously selected according to the therapeutic objective.

[0194]

For example, a pharmaceutical preparation containing the gene transfer vector of the invention can be provided in the form of said vector entrapped in liposomes or in the form of cultured cells infected with a virus containing a retrovirus vector harboring the object gene.

[0195]

These can be prepared as solutions in phosphate-buffered saline (pH 7.4), Ringer's injection or an intracellular fluid composition injection, for instance, or in such a form that it may be administered together with a substance capable of enhancing the efficiency of gene transfer, such as protamine.

[0196]

The method of administering the above pharmaceutical preparation is not particularly restricted but is selected according to the dosage form, the

patient's age, sex and other factors, the severity of illness, and other conditions.

[0197]

The amount of the active ingredient to be
5 incorporated in said pharmaceutical composition or
preparation are not particularly restricted but can be
liberally selected from a broad range according to the
desired therapeutic effect, administration method,
duration of treatment, the patient background inclusive of
10 age and sex, and other conditions.

[0198]

Generally speaking, the daily dose of the gene-
harboring retrovirus vector as a pharmaceutical
preparation per kilogram body weight may for example be
15 about 1×10^3 pfu to 1×10^{15} pfu in terms of retrovirus titer.

[0199]

In the case of cells to which the object gene
has been introduced, the dosage may be judiciously
selected from the range of about 1×10^4 cells/body to about
20 1×10^{15} cells/body.

[0200]

The pharmaceutical product can be administered
once a day or in a few divided doses a day, and may be
administered intermittently, for example one to several
25 weeks apart. Moreover, it can be advantageously

administered in combination with a substance capable of enhancing the efficiency of gene transfer, such as protamine, or a pharmaceutical preparation containing said substance.

5 [0201]

When the gene therapy of the present invention is applied to the treatment of a cancer, the various methods of gene therapy mentioned above may be used in a suitable combination (combination gene therapy) and/or in
10 combination with the conventional cancer chemotherapy, radiation therapy, immunotherapy and/or other therapy. Furthermore, the gene therapy according to the present invention can be carried out with reference to the NIH Guidelines, inclusive of the safety aspect thereof [cf.
15 Recombinant DNA Advisory Committee, Human Gene Therapy, 4, 365-389 (1993)].

[0202]

In accordance with the present invention, the presence of a mutant p51 gene which promotes tumorigenesis
20 in human cells can be detected by the procedure which comprises preparing a blood, serum or other biological sample, optionally extracting the nucleic acid, and analyzing it for the presence or absence of a sensitive mutant p51 gene. Furthermore, in accordance with the
25 present invention, the existence of a neoplastic change in

cells or tissues, a marker of progression to a prodromal state of malignancy or a prognostic marker can be detected by the procedure which comprises preparing a disorder-containing biological sample and analyzing it for the presence or absence of a neoplastic mutant p51 gene. By the above procedure, the presence of a neoplasm in cells or tissues, a marker of progression to a prodromal state of malignancy or a prognostic marker can be detected, thus making it possible to establish a diagnosis, for example the diagnosis of a cancer, evaluate the effect of a cancer therapy, or predict the prognosis of the cases.

[0203]

According to this detection method, based on the mutant p51 gene information obtained from a tumor-bearing patient sample, for example on the information about the mutation site of the p51 gene and the mutant sequence information, the relevant mutant DNA fragment is prepared and designed so that it may be used in the screening for the mutant gene and/or the amplification thereof. More particularly, the probe for use in plaque hybridization, colony hybridization, Southern blotting, Northern blotting, etc. or the probe for amplification of the mutant DNA fragment by polymerase chain reaction (PCR) to amplify the nucleotide sequence with polymerase can be constructed. For such purposes, a primer having the same sequence as

the mutation is first prepared and reacted, as a screening probe, with a biological sample (nucleic acid sample), whereby the presence of a gene having a mutated p51 gene sequence can be confirmed. To facilitate detection of the target sequence, said nucleic acid sample may be prepared by utilizing various techniques such as lysis, restriction enzyme digestion, electrophoresis or dot blotting.

[0204]

Referring to the screening method mentioned above, the use of a PCR method is particularly preferred from the standpoint of sensitivity, and this method is not particularly restricted inasmuch as the mutant p51 fragments are used as primers. Thus, any of the hitherto-known techniques [Science, 230, 1350-1354 (1985)] and versions of PCR which have been newly developed or will be used in future [Yoshiyuki Sakaki et al. (ed): Experimental Medicine, Supplemental Issue, 8(9) (1990), Youdo-sha; Protein, Nucleic Acid and Enzyme, Special Supplemental Issue, 35(17) (1990), Kyoritsu Shuppan] can be employed.

[0205]

The DNA fragments for use as primers are chemically synthesized oligoDNAs, and these oligoDNAs can be synthesized by using an automated DNA synthesizing hardware, such as the DNA synthesizer Pharmacia LKB Gene Assembler Plus (Pharmacia). The length of the primer so

synthesized (sense primer or antisense primer) is preferably the equivalent of about 10-30 nucleotides. The probe for use in said screening is usually labeled but may be an unlabeled one, and the detection may be made either
5 directly or indirectly by specific binding with a labeled ligand. The suitable label and the method of labeling the probe or the ligand are known to those skilled in the art, and the radioactive label, biotin, fluorescent group, chemiluminescent group, enzyme, antibody, etc. which can
10 be incorporated by the known techniques such as nick translation, random priming or kinase treatment are also included in the relevant technology.

[0206]

The PCR method for use in said detection
15 includes RT-PCR, for instance, and various modifications of PCR which are used in the art can be applied likewise.

[0207]

It is also possible to detect the wild type p51 gene and/or mutant p51 gene and quantitate the DNAs of
20 these genes. This technology includes but is not limited to the competitive assay such as MSSA [Kinoshita, M. et al., CCA, 228, 83-90 (1994)] and PCR-SSCP which is known to be a mutation detecting technique utilizing the change in mobility associated with a change in the higher-order
25 structure of a single-stranded DNA [Orita, M. et al.,

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Genomics, 5, 874-879 (1989)].

[0208]

The above analytical methods mentioned by way of example can be carried out as follows. For example, one or a plurality of primers containing the mutation of p51 (e.g. the mutated sequence based on site mutation information obtained from a cancer patient or the like) are first prepared and hybridized with the DNA obtained from a biological sample. Then, the mobility and peak area measured by SSCP analysis of the standard wild type p51 DNA fragment are compared with the mobility and peak area in the test sample as the product of amplification using said primers to thereby detect the mutation in a specific region of p51 and simultaneously quantitate the product of mutation.

[0209]

The test sample containing the mutant p51 gene (DNA) to be measured is not particularly restricted inasmuch as it contains said mutant gene, thus including various biological materials such as blood, serum, urine and excised tissues. The mutant p51 DNA can be extracted from such test samples, purified and prepared in the routine manner. Therefore, by comparing the mobility of said standard DNA fragment of the invention, as determined in advance, with the mobility of the amplification product

In the test sample as obtained in the PCR amplification of the p51 DNA of the test sample using a mutant p51 primer pair, the mutation in a specific region of p51 DNA can be detected expediently and accurately.

5 [0210]

Furthermore, when standards established in known steps of quantity are used, the quantitation of the mutant p51 in the test sample can be made at the same time by comparing the peak areas of the standards with the peak area of the amplification product of p51 DNA in the test sample in the PCR amplification step using the mutant primer set mentioned above. The primer set, standards, PCR-SSCP analysis and detection means can be liberally modified by those skilled in the art and the present invention encompasses such modifications, of course, inasmuch as the sequences of the wild type p51 gene and mutant p51 genes are employed.

[0211]

The above assay technology according to the present invention is now described more specifically. To begin with, the DNA is extracted from the serum of a cancer patient by the routine procedure such as alkali or acid treatment. Then, a primer set comprising a minus chain partial sequence of a defined length consisting in a part of the nucleotide sequence (145-1487) shown in Figs.

2-8 and a plus chain partial sequence of a defined length consisting in a part of the fluorescent-labeled nucleotide sequence (145-1487) as well as a heat-resistant DNA polymerase are caused to act on the DNA solution obtained
5 above to amplify the labeled DNA fragment. On the other hand, one or a plurality of DNA fragments containing a mutant sequence chemically synthesized according to the p51 site mutation information obtained from, for example, a cancer patient are respectively integrated in plasmid
10 vectors and E. coli is transformed. After mass culture and purification, the purified recombinant plasmids are used to prepare e.g. 10^3 copy, 10^4 copy, 10^5 copy, 10^6 copy, 10^7 copy and 10^8 copy standards. Said primer set comprising a minus chain partial sequence consisting in a
15 defined partial sequence of said nucleotide sequence (145-1487) shown in Figs. 2-8 and a plus chain partial sequence consisting in a defined partial sequence of the fluorescent-labeled nucleotide sequence (145-1487) shown
in Figs. 2-8 as well as a heat-resistant DNA polymerase to
20 amply the labeled DNA fragment. The solution of DNA amplified above is heated at about 95°C for about 5 minutes, then immediately cooled on ice, and a SSCP analysis is performed using an automatic sequencer, such as ALF Automatic Sequencer (Pharmacia), whereby the
25 fluorescent peak can be detected. Phoresis in this SSCP

analysis is performed preferably at about $30^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

[0212]

The peak (mobility) of the DNA obtained from the patient's serum is compared with the peaks (mobilities) of the standards and the peak in agreement with a standard is
5 ascertained from the migration time. In this manner, the type (kind) of mutation of the patient p51 can be ascertained. Moreover, by calculating the peak areas of standards and constructing a standard curve, the p51DNA
10 can be quantitated from the calculated peak area of the patient's DNA.

[0213]

The present invention, therefore, provides an expedient test protocol for the concurrent detection and
15 quantitation of mutation in a given region of p51 DNA in the test sample through the above measurement.

[0214]

The assay method of this invention can be carried out conveniently by utilizing a reagent kit for
20 detecting the wild type p51 gene and mutant p51 gene in a sample.

[0215]

Therefore, the present invention further provides a reagent kit for detection of wild type p51 and
25 mutant p51 characterized by its comprising said wild type

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p51 DNA fragment and mutant p51 DNA fragment.

[0216]

This reagent kit, inasmuch as it contains a DNA
fragment capable of hybridizing with a part or the whole
5 of the nucleotide sequence (145-1487) shown in Figs. 2-8
or its complementary oligonucleotide sequence or a DNA
fragment capable of hybridizing with a part or the whole
of a mutant sequence of the nucleotide sequence (145-1487)
shown in Figs. 2-8 or a nucleotide sequence complementary
10 to said sequence, may contain other components such as a
labeling agent, reagents essential to PCR (e.g. TaqDNA
polymerase, deoxynucleotide triphosphate, primers, etc.).

[0217]

As the labeling agent, a radio isotope or a
15 chemical modifier such as a fluorescent substance can be
employed, although the DNA fragment itself may have been
conjugated with the labeling agent in advance. Moreover,
this reagent kit may further comprise a suitable reaction
diluent, standard antibody, buffer, washing solution,
20 reaction stopper, etc. for convenience in carrying out an
assay.

[0218]

The present invention further provides a method
of diagnosis using said assay technique and a diagnostic
25 agent and a diagnostic kit for use in said method of

-89-

diagnosis.

[0219]

Further, by direct or indirect sequencing of the mutant p51 sequence obtained from the test sample by the
5 above procedure, it is possible to discover novel p51-related genes having high homology to the wild type p51.

[0220]

The present invention, therefore, further provides a method of screening for human p51-related genes
10 in test samples through the above-described assay and sequencing of mutant p51 DNA in such test samples.

[0221]

Furthermore, the wild type p51 and/or mutant p51 can be identified by synthesizing the protein encoded by
15 the human p51 gene shown in Fig. 1 or the protein corresponding to the amino acid sequence derived from the sequence shown in Fig. 1 by deletion, substitution or addition of one or a plurality of amino acids or a partial sequence thereof, or synthesizing the antibody against any
20 of such proteins.

[0222]

Therefore, the present invention provides a method for assay of antibodies against wild type p51 and/or mutant p51 and for assay of the antigen. By this
25 assay method, the degree of neoplastic disturbance or the

malignancy of a malignant tumor can be estimated based on the change in the wild type p51 polypeptide. The change mentioned above can be determined by p51 sequence analysis by said routine technology but more preferably the change
5 in the p51 polypeptide or the presence or absence of the p51 protein is detected using an antibody (a polyclonal antibody or a monoclonal antibody). A specific example of the assay method of the invention is as follows. With the p51 antibody, the p51 protein can be immunoprecipitated
10 from a solution containing a human biological material isolated from a human being, such as blood or serum, and the antibody can be reacted with the p51 protein on a polyacrylamide gel Western blot or immunoblot. Moreover, with the p51 antibody, the p51 protein in a paraffin
15 section or frozen tissue section can be detected by an immunohistochemical technique. The technology for antibody production and purification is well known in the art and the known techniques can be selectively employed.

[0223]

20 The preferred specific modes of practicing the method of detecting the wild type p51 or a mutant thereof include enzyme-linked immunosorbent assay (ELISA) inclusive of the sandwich technique using a monoclonal antibody and/or a monoclonal antibody, radioimmunoassay
25 (RIA), immunoradiomatrix assay (IRMA) and

immunoenzymematrix assay (IEMA).

[0224]

Furthermore, in accordance with the present invention, it is also possible to provide a cell membrane
5 fraction having p51-binding activity for the p51 protein or the p51 receptor present on the cell surface. To acquire said p51 receptor, the labeled p51 protein is conjugated in a biological sample containing the cell membrane fraction, the resulting p51 conjugate is isolated
10 by extraction and purified, and the amino acid sequence of the isolate is determined. The acquisition and sequencing of this p51 receptor protein fall within the expertise of one skilled in the art.

[0225]

15 The present invention can be applied to the screening for compounds (p51 receptor reaction products: the compound may be a low molecular compound, a high molecular compound, a protein, a protein fragment, an antigen, an antibody or the like) by using the p51
20 receptor polypeptide or a binding fragment thereof for the screening of various drugs. Preferably, the p51 receptor protein is utilized. The p51 receptor polypeptide or its fragment for use in such a screening test may be immobilized on a solid support or used in the form of a
25 suspension in a fluid carried to the cell surface. To

mention an example of drug screening, host eucaryotic or procaryotic cells transformed stably with a recombinant polypeptide and expressing the polypeptide or its fragment can be utilized, preferably in a competitive binding assay.

5 Moreover, such cells in the free or immobilized state can be used in a standard binding assay. More preferably, the formation of a complex between the p51 receptor polypeptide or its fragment and a test substance is quantitated to detect the degree of inhibition of said
10 formation of a complex between the p51 receptor polypeptide or fragment and the p51 polypeptide or fragment by the test substance is detected, whereby the screening for a compound can be accomplished.

[0226]

15 Thus, the present invention provides a method of drug screening characterized by contacting such a substance with the p51 receptor polypeptide or a fragment thereof by a per se known technique and, then, detecting the presence of a complex between said substance and said
20 p51 receptor polypeptide or fragment or the presence of a complex between said p51 receptor polypeptide or fragment and a ligand. Further, the p51 receptor activity is measured to see whether said substance may antagonize the p51 receptor to exhibit the p51 activities defined
25 hereinbefore, for example the activity to modify the cell

cycle or modulate the induction of apoptosis.
Specifically, in carrying out such a competitive binding
assay, the p51 receptor polypeptide or its fragment is
labeled. The free p51 receptor polypeptide or fragment is
5 separated from the protein-protein complex. Then the
amount of the free label (non-complex-forming) can be a
measure of the binding between the factor to be assayed
and the p51 receptor or inhibition of the p51 receptor-p51
polypeptide binding. The small peptide (pseudopeptide) of
10 the p51 polypeptide is thus analyzed, and the one having
p51 receptor inhibitory activity is determined.

[0227]

Another drug screening method of the present
invention is a method of screening for compounds having an
15 adequate binding affinity for the p51 receptor polypeptide.
Briefly, a large number of different peptide test
compounds are synthesized on solid supports such as
plastic pins or other surfaces. Then, the peptide test
compounds are reacted with the p51 receptor polypeptide,
20 followed by washing. Then, the reacted and bound p51
receptor polypeptide is detected by a per se known
technique [PCT: W084-03564]. The purified p51 receptor
can be directly coated on a plate for use in said drug
screening. However, the p51 receptor polypeptide can be
25 immobilized on a solid phase by antibody supplementation

using a non-neutralizing antibody against the polypeptide. Furthermore, the present invention is directed to use of a competitive drug screening assay in which a neutralizing antibody specifically binding the p51 receptor polypeptide
5 and a test compound are caused to compete with each other for the binding to the p51 receptor polypeptide or fragment. By this competitive assay using the antibody, the presence of peptides having one or more epitopes or antigenic determinant sites of the p51 receptor
10 polypeptide can also be detected.

[0228]

Referring, further, to drug screening, a still another method comprises the use of a host eucaryotic cell line or cells containing a nonfunctional p51 gene. Thus,
15 after the host cell line or cells are allowed to grow in the presence of a drug compound for a predetermined time, the proliferation rate of the host cells is measured to see whether the compound may modulate apoptosis or the cell cycle. As a means for measuring the proliferation
20 rate, it is possible to measure the biological activity of the p51 receptor.

[0229]

Moreover, in accordance with the present invention, for the purpose of developing the more active
25 or stable p51 polypeptide derivatives or drugs which will

potentiate or interfere with the function of the p51 polypeptide in vivo, various interactive biologically active polypeptides or structural analogs, e.g. p51 agonists, p51 antagonists, p51 inhibitors, etc., can be constructed. Such structural analogs can be characterized by, for example, analyzing the three-dimensional structures of complexes between p51 and other proteins by X-ray crystallography, computer modeling or a combination of such techniques. Furthermore, the information on structural analogs can also be generated by protein modeling based on the structures of homologous proteins.

[0230]

The method of obtaining the still more active or stable p51 polypeptide derivatives may for example involve an alanine scan analysis. In this method, Ala is substituted for each amino acid residue and the effect of substitution on peptide activity is determined. Each amino acid residue in a peptide is thus analyzed and the region or regions of importance to the activity or stability of the peptide are determined. By using this method, the more active or stable p51 derivatives can be designed.

[0231]

It is also possible to isolate the target-specific antibody selected by a functional assay and

analyze its crystal structure. As a rule, a pharmacore providing the basis for further drug design can be obtained by this approach. It is possible to identify or isolate a peptide from a chemically or biologically constructed peptide bank by causing formation of an anti-
5 ideotype antibody against a functional pharmacoactive antibody. Therefore, the selected peptide is also expected to serve as a pharmacore.

[0232]

10 In this manner, drugs having improved p51 activity or stability or drugs acting as inhibitors, agonists or antagonists of p51 activity can be designed and developed.

[0233]

15 In accordance with the cloned p51 sequence, a sufficient amount of p51 polypeptide can be procured and submitted to X-ray crystallographic and other analytical research. Furthermore, the p51 protein having the amino acid sequence shown in Fig. 1 as provided by the present
20 invention enables establishment of a computer modeling program which may take the place of X-ray crystallography or supplement the latter technique.

[0234]

25 Furthermore, by constructing a human p51 gene-bearing knockout mouse (mutant mouse) in accordance with

the present invention, it is possible to detect which sites of the human p51 gene sequence will influence said various p51 activities in vivo, that is to say what functions the p51 gene product and mutant p51 gene products will have in vivo.

[0235]

This is a technology to intentionally modify the genetic information of organisms by utilizing homologous recombinations of genes, and the method using mouse embryonic stem cells (ES cells) can be mentioned as an example [Capeccchi, M. R. Science, 244, 1288-1292 (1989)].

[0236]

The method of constructing such mutant mice as above is well known to those skilled in the art, and by applying the human wild-type p51 gene or mutant p51 gene of the present invention to the above technology as modified (Noda, T. (ed.), Experimental Medicine, Supplemental Issue, 14(20) (1996), Youdo-sha), mutant mice can be easily established. Therefore, by utilizing the above technology, drugs having improved p51 activity or stability or drugs acting as inhibitors, agonists or antagonists of p51 activity can be designed and developed.

[0237]

The present invention includes the following:

[1]. A gene coding for the following protein (a) or (b):

(a) a protein having an amino acid sequence shown in Fig.
1

(b) a protein having an amino acid sequence derived from
the amino acid sequence shown in Fig. 1 by deletion,
5 substitution or addition of one or a plurality of amino
acids and having p51 activity.

[0238]

[2]. A gene comprising the following DNA (a) or (b):

(a) a DNA having a nucleotide sequence identified by the
10 nucleotide numbers 145-1487 of the nucleotide sequence
shown in Figs. 2-8;

(b) a DNA capable of hybridizing with the DNA having a
nucleotide sequence identified by the nucleotide numbers
145-1487 of the nucleotide sequence shown in Figs. 2-8
15 under stringent conditions and coding for a protein having
p51 activity.

[3]. A human p51 gene having the nucleotide sequence
shown in Figs. 2-8.

[0239]

20 [4]. A cloned cDNA coding for the following protein (a)
or (b):

[0240]

(a) a protein having an amino acid sequence shown in
Fig. 1;

25 (b) a protein having an amino acid sequence derived

from the amino acid sequence shown in Fig. 1 by deletion, substitution or addition of one or a plurality of amino acids and having p51 activity.

[0241]

- 5 [5]. A cloned cDNA comprising the following DNA (a) or (b):

(a) a DNA having a nucleotide sequence identified by the nucleotide numbers 145-1487 of the nucleotide sequence shown in Figs. 2-8;

- 10 (b) a DNA capable of hybridizing with a DNA having a nucleotide sequence identified by the nucleotide numbers 145-1487 of the nucleotide sequence shown in Figs. 2-8 under stringent conditions and coding for a protein having p51 activity.

15 [0242]

[6]. A DNA characterized in that it is capable of the hybridizing with a nucleotide sequence identified by the nucleotide numbers 145-1487 of the nucleotide sequence shown in Figs. 2-8 under stringent conditions.

- 20 [7]. The DNA defined in [6], which is used as a primer or a probe.

[0243]

[8]. A vector harboring the gene (including alleles of p51 gene) defined in any one of [1] to [4].

- 25 [9]. A host cell transformed with the vector defined in

[6].

[0244]

[10]. A protein defined below in (a) or (b):

(a) a protein having an amino acid sequence shown in Fig.

5 1;

(b) a protein having an amino acid sequence derived from the amino acid sequence shown in Fig. 1 by deletion, substitution or addition of one or a plurality of amino acids and having p51 activity.

10 [11]. The protein defined in [10], which is a gene
product of the gene defined in any one of [1] to [3] or a
recombinant protein obtained by recombinant DNA technology.

[0245]

[12]. A method for producing the protein defined in [10],
15 which comprises growing the host cell defined in [9] in a
culture medium and harvesting a protein from the resulting
culture.

[0246]

[13]. A method of inhibiting tumorigenesis which
20 comprises transferring the cloned p51 cDNA to tumor cells.

[14]. A method of inhibiting tumorigenesis which comprises transferring the p51 protein product to tumor cells.

[0247]

25 [15]. A pharmaceutical preparation comprising the p51

gene or its allele.

[16]. A pharmaceutical preparation comprising the p51 protein.

[17]. A pharmaceutical preparation comprising the
5 recombinant p51 protein.

[18]. A drug for gene therapy which comprises the p51 gene or its allele as an active ingredient.

[19]. A pharmaceutical composition comprising the p51 protein as an active ingredient.

10 [0248]

[20]. A pharmaceutical composition comprising the p51 protein as an active ingredient.

[21]. A method of screening for p51- or p53-related genes which comprises using the gene according to any one of
15 items [1] to [5].

[0249]

[22]. A cancer diagnostic reagent comprising the gene defined in any one of [1] to [5].

[23]. A cancer diagnostic reagent comprising the protein
20 defined in [10] or [11].

[0250]

[24]. A method of screening for inhibitors of cell tumorigenesis which comprises using the gene defined in any one of [1] to [6].

25 [25]. An inhibitor which is capable of inhibiting cell

tumorigenesis and obtained by the screening method defined in [24].

[0251]

[Effects of the invention]

5 In accordance with the present invention, there is provided a novel human p51 gene which is of value as a cell proliferation suppressive gene. The novel gene of the present invention resembles the gene coding for the p53 protein or p73 protein. Therefore, the gene of the
10 invention can be utilized in studies on the relationships of the analyzed functions of these related genes to various diseases and used in studies for application to gene diagnosis and the medicinal use of these genes in various diseases. Moreover, by using the gene of the
15 invention, the expression pattern of the gene in various human tissues can be explored and its functions in vivo can be analyzed.

[0252]

 In addition, with this gene, the human p51
20 protein encoded by the gene can be produced in large quantities by the genetic engineering technology. Thus, the gene and gene fragments provided by the present invention can be integrated with expression vectors to construct recombinant human p51 proteins and study p51
25 protein activity and the functions, e.g. binding activity,

of the p51 protein.

[0253]

Furthermore, the p51 protein is useful for the pathological elucidation, diagnosis and therapy of diseases associated with the p51 gene or its product (e.g. diseases related to the transcription activity of cells and various diseases related to apoptosis, particularly cancers).

[0254]

10 The p51 protein has actions (physiological functions) similar to those of the p53 protein. Therefore, when various biological stresses such as virus infection, cytokine stimulation, hypoxia, a change in the nucleotide pool, drug-induced metabolic derangements, etc. act upon
15 the living tissues, the protein of the invention exhibits such functions as signal transductions through interactions with other proteins and transcriptional control over the other genes to thereby modulate replication of the cell DNA in the tissues subjected to
20 said biological stresses, interrupt their cell cycle to repair the cells, eliminate cells through apoptosis, or promote the differentiation of cells to thereby control to the defense of living tissues against said stresses.

[0255]

25 In accordance with the present invention, there

are provided a gene transfer vector containing the human p51 gene or an allele thereof which is useful for gene therapy, cells into which said p51 gene or allele has been introduced, a gene-therapeutic agent containing said
5 vector or cells as an active ingredient, and a method of gene therapy exploiting them.

[0256]

Furthermore, in accordance with the present invention, there is provided a pharmaceutical product
10 containing the p51 protein as an active ingredient which has activity to suppress growth of various cancer cells and finds application in the treatment of various neoplastic diseases and associated symptoms through said activity.

15 [0257]

[Examples]

The present invention will be described below in more detail with reference to Examples.

[0258]

20 Example 1 Isolation of the human p51 gene

(1) Cloning and DNA sequencing of the human p51 gene

(a) The present inventors carried out a PCR amplification using the following p73-F1 sense primer and p73-R1 antisense primer and then a second amplification by a
25 nested PCR using the following p73-F2 sense primer and

- 105 -

p73-R2 antisense primer.

[0259]

p73-F1: 5'-TA(CGT)GCA(CGT)AAA(G)ACA(CGT)TGC(T)CC-3'

p73-R1: 3'-TGC(T)GCA(CGT)TGC(T)CCA(CGT)GGA(CGT)A(C)G-5'

5 p73-F2: 5'-TA(CGT)ATA(CT)A(C)GA(CGT)GTA(CGT)GAA(G)GG-3'

p73-R2: 3'-ATGAAC(T)A(C)GA(CGT)A(C)GA(CGT)CCA(CGT)AT-5'

More specifically, from the human skeletal muscle polyA+RNA (Clontech), the cDNA was synthesized using a random primer and an oligo dT primer. Then, a cDNA library consisting of about 10^7 plaques as constructed using λ ZipLox (Gibco BRL) as the vector was amplified and the DNA was extracted. Using 0.2 μ g of the cDNA as the template and said p73-F1 and p73-R1 as primers, an amplification reaction was carried out in 25 cycles of 94°C, 30 sec., 45°C, 30 sec. and 72°C, 30 sec in accordance with the Tag Polymerase (Gibco-BRL) manual. Then, using 1/100 of the amplification product as the template and said p73-F2 and p73-R2 as primers, a further amplification was carried out under the same conditions.

20 Since a band of 172 bp as deduced from the structure of
the p53 gene was obtained, a restriction enzyme cleavage
map of the band was prepared. As a result, the presence
of a gene other than the p53 gene was detected. This band
was subcloned in pGEM7 (Promega) and using ABI377
25 Automatic Sequencer (ABI), the nucleotide sequence was

determined in the routine manner. As a result, it was found to be a DNA fragment derived from a novel gene which resembles the p53 gene but has a different novel nucleotide sequence.

5 [0260]

Separately, a similar analysis was carried out using cDNA libraries derived from other organs (e.g. brain). As a result, a DNA fragment derived from a further different novel gene resembling the p53 gene was detected but it was found to be a fragment derived from the p73 gene.

[0261]

This subcloned DNA fragment was excised and using the BcaBest labeling kit (Takara), a labeled probe was constructed. The plaque hybridization assay of an unamplified library of 2.4×10^6 plaques as constructed using the oligo dt primer alone in otherwise the same manner as in the construction of said cDNA library gave 8 positive clones. Since λ ZipLox can be easily converted to a plasmid using the Cre-LoxP system, sequencing of the plasmid obtained by conversion was carried out in the routine manner using LICOR's automatic sequencer and ABI377 automatic sequencer (ABI).

[0262]

25 Then, between the nucleotide sequence of the

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gene obtained and the nucleotide sequences of the p53 and p73 genes, a homology search was made with FASTA Program using GCG software (Wisconsin Sequencing Package, Genetics Computer Group) [Person, W. R. and Lipman, D. J., Proc.

5 Natl. Acad. Sci. U.S.A., 85, 1435-1441 (1988)].

[0263]

As the result of said homology search, one of the clones selected by the above method and sequenced were found to have high homology with respect to the p53 gene
10 and p73 gene. The molecular masses calculated from the deduced amino acids encoded by the gene sequence of the one clone were 50,894 Da.

[0264]

This clone was named p51 by the present
15 inventors.

[0265]

The full-length nucleotide sequence of the gene possessed by the clone obtained above is shown in Figs. 2-8. In the 145th ~ 1487th position, the nucleotide
20 sequence was found to have an open reading frame coding for the amino acid sequence (448 amino acids) shown in Fig. 1, namely, a nucleotide sequence (1344 nucleotides) identified by nucleotide numbers 145~1487 of the nucleotide sequence shown in Figs. 2-8. Moreover, the
25 deduced amino acid sequence encoded by the nucleotide

sequence of the gene possessed by this clone had a transcriptional activation domain in the 1st ~ 59th position, the DNA binding domain in the 142nd ~ 321st position, and the oligomerization domain in the 359th ~ 397th position.

[0266]

The amino acid sequence encoded by the gene (human p51 gene) of the invention was compared with the amino acid sequences of the p53 protein and p73 β protein for homology comparison among the three sequences (Fig. 9). In the diagram, the amino acids common to the 3 sequences are boxed.

[0267]

Fig. 10 is a schematic diagram showing features of the structural domains of the p51 protein along with those of the p53 protein and p73 β protein. In the diagram, "TA" represents a transcriptional activation domain, "DNA binding" represents a DNA-binding domain, and "Oligo" represents an oligomerization domain. The structural features of the p51 protein and p73 protein were deduced from the structural features of the p53 protein.

[0268]

As a result, the homology of the deduced amino acid sequences of the p51 protein, p53 protein and p73 protein in each of full-length sequence, transcriptional

activation domain, DNA-binding domain, and oligomerization domain was respectively as follows: between p51 protein and p53 protein, 36%, 22%, 60% and 37%, respectively; between p51 protein and p73 protein, 42%, 30%, 87% and 65%,
5 respectively; and between p53 protein and p73 protein, 28%, 27%, 63% and 83%, respectively (see Table 1).

[0269]

Moreover, although the 448 amino acid residue-structure of the p51 protein was shorter than the 636
10 amino acid residue-structure of the p73 α protein, the total structure of the p51 protein resembled the p73 protein with the C-terminal region split off.

[0270]

These results indicated that although the
15 putative amino acid sequence of the p51 protein resembles the deduced sequences of both the p53 protein and p73 protein, its homology to the amino acid sequence of the p73 protein is higher than its homology to the p53 protein and that the homology between p51 protein and p73 protein
20 is higher than the homology between p53 protein and p73 protein. Furthermore, between p51 protein and p73 protein, a homology was found in the region where no homology was found between p53 protein and p73 protein or between p53 protein and p51 protein. These results suggest that, on
25 the amino acid sequence level, the p51 protein can be said

to be closer to the p73 protein than to the p53 protein.

[0271]

Example 2 Confirmation of p51mRNA expression in normal human tissue

5 (1) Northern blot analysis

Expression of p51mRNA in normal human tissue was assessed by Northern blotting using, as the probe, a human cDNA clone labeled by the random oligonucleotide priming method.

10 [0272]

Northern blot analysis was carried out using Human Multiple Tissue Northern Blot (Clontech, Palo Alto, Calif., U.S.A.) in accordance with the product manual.

[0273]

15 Thus, the EcoRI fragment (600 bp: corresponding to the 5' end of cDNA) of a PCR amplification product of the DNA clone obtained in Example 1 was labeled with [³²p]-dCTP (Random Primed DNA Labeling Kit, Boehringer Mannheim) for use as a probe.

20 [0274]

Blotting was made using ExpressHyb Hybridization Solution (Clontech) under the conditions directed in the user manual, and detection was made using BAS2000 (FUJI).

[0275]

25 The results are shown in Fig. 11 and Fig. 12.

Fig. 11 shows the result of Northern hybridization carried out with the filter purchased from Clontech. Fig. 12 shows the result of Northern hybridization carried out with a filter constructed by the present inventors using the RNA purchased from Clontech. Fig. 11 shows the electrophoretogram with 2 μ g poly A-RNA added for each lane, and Fig. 12 is the electro-phoretogram with 0.5 μ g poly A+RNA added for each lane.

[0276]

The lanes in Fig. 11 represent the result for 1: heart, 2: brain, 3: placenta, 4: lung, 5: liver, 6: skeletal muscle, 7: spleen, and 8: pancreas. The lanes in Fig. 12 represent the result for 1: mammary gland, 2: prostate, 3: salivary gland, 4: stomach, 5: thymus, 6: thyroid, 7: trachea, and 8: uterus.

[0277]

It was found that the distribution of expression of the mRNA (4.4 kb) of the gene named "human p51 gene" according to the present invention was rather confined in contrast to the ubiquitous expression of p53 mRNA, with the expression level being highest in skeletal muscle, seconded by placenta, and decreased progressively in placenta, trachea, mammary gland, prostate, salivary gland, thymus, uterus, stomach, lung, brain and heart in the order mentioned. In other tissues (e.g. adrenal gland,

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small intestine, spinal cord, spleen), no expression of p51mRNA could be detected.

[0278]

The expression pattern of the p73 gene is also
5 tissue-restricted. However, it was found that while the
expression of p51 gene overlapped the expression of the
p73 gene (expression in the same tissue), the distribution
of expression was broader than the distribution of
expression of the p73 gene.

10 [0279]

The above difference in expression tissue
distribution among the human p51 gene, p53 gene and p73
gene suggested that notwithstanding the resemblance in
biological activity among these genes, they are dissimilar
15 in function depending on tissues in vivo.

[0280]

Further research also revealed that, in various
human tissues, the p51mRNA, as in the case of p73 protein,
exists in selectively spliced forms (alternative splicing
20 variants), namely a short form encoding the p51A protein
and a long form encoding the p51B protein. The latter
long form encoding p51B was found to have homology to the
factor named "ket" which had been accidentally discovered
in a search for the glutamate receptor of the tongue. The
25 3 kb mRNA, which is a main transcript in skeletal muscle,

was the most abundant mRNA observed in all the tissues investigated. The short-form cDNA clone was suspected to be derived from this transcript. Interestingly, in contrast to the mRNA observed in normal tissues, this
5 short-form of p51mRNA was found to have been expressed in many tumor cell lines.

[0281]

Fig. 13 is a schematic diagram comparing the structures of the respective alternative splicing variants
10 of the p51 protein and p73 protein. This p51BmRNA encoded a protein having a molecular mass (calculated) similar to that of p73 β . Functional differences between p51A and p51B remain unknown.

[0282]

15 Example 3 Localization of the p51 clone and chromosome by the direct R-banding FISH and radiation hybrid method

A clone presumably derived from unspliced RNA was found among cDNA clones. Prepared from the nucleotide sequence of the clone was a 20mer primer amplifiable by
20 PCR in intron 8 in a human genome-specific manner (not amplified from hamster genome), which was then analyzed according to Genebridge4 user's manual.

[0283]

As a result, the p51 gene was mapped on the
25 chromosome band 3q26. According to the radiation hybrid

method, the p51 gene was localized in the region from AFBM327YD9 to 5-66cR between the two markers AFBM327YD9 and WI-1189 on the chromosome.

[0284]

- 5 Example 4 Mutation of p51 in various human cancer cell lines and human tumors

The most intriguing question about the p51 gene is the question of whether the features of the p53 tumor suppressor gene are shared by the p51 gene as well as the relationship of a mutation of the present gene with the morphogenesis of a human tumor. Therefore, using various tumor cell lines, a search was made for the presence or absence of mutation of the p51 gene. For this search, the method for functional analysis of separated alleles in yeasts (FASAY), which was previously used by the present inventors in the identification and characterization of p53 mutation, was used [Ishioka et al., Nat. Genet. 5, 124-129 (1993)].

[0285]

- 20 A complementary full-length DNA fragment coding for the human p51 gene was amplified by the same PCR method as used in the determination described hereinbefore to acquire the nucleotide sequence of the amplification product covering the full-length sequence encoding the p51 gene and this nucleotide sequence was determined by direct
- 25

sequencing to detect the presence or absence of a mutation.

[0286]

Tumor cells were respectively cultured in Dulbecco's Modified Essential Media supplemented with 10% fetal calf serum in a 5% CO₂ environment. Since all the p51AcDNA clones could amplify the p53cDNA in the previous analysis, the quality of cDNAs of cell lines was guaranteed.

[0287]

Of 102 cell lines, 67 lines analyzed were capable of amplifying the p51 DNA fragment. The nucleotide sequence was determined by direct sequencing for 35 of the above cell lines.

[0288]

Mutations were found in two cell lines, namely Ho-1-u-1 (JCRB0828), which is a head-and-neck cancer cell line, and SKG-IIIa (JCRB0611), which is a cervical cancer cell line.

[0289]

The mutation was Ser145 → Leu in the former and Gln165^s → Leu in the latter. With regard to the p53 protein, it was likely that the normal function of the p53 protein had been defected by mutation in the former and by human papilloma virus infection in the latter. Moreover, in the mRNAs derived from tumor cells, various splicing

variants were noted.

[0290]

Referring to human primary cancers, the nucleotide sequences of the products obtained by SSCP and
5 RT-PCR techniques were determined by direct sequencing in search for p51 gene mutation. In a total population of 66 human tumor cases, namely, 8 neuroblastoma cases, 8 colon cancer cases, 8 breast cancer cases, 8 lung cancer cases, 8 brain tumor cases, 8 esophageal cancer cases, 8
10 hepatocellular cancer cases, 6 pancreatic cancer cases, and 4 renal cancer cases, a mutation of Ala145 → Pro was detected in one lung cancer case.

[0291]

The analysis of the above 3 cases was invariably
15 an analysis of cDNA and it was clear that the expression originated from a single chromosomal locus.

[0292]

Experimental Example 1 Suppression of colony formation by p51 transformation

20 The p53 protein has an ability to block cells in the G1 phase or induce apoptosis.

[0293]

To investigate the colony formation inhibitory activity of the p51 protein of the invention, the SAOS2
25 osteosarcoma cell line (accession number: ATCC HTB85) was

co-transfected with a puromycin-resistant expression plasmid (pBABEpuro: Morgenstern J. Nuc. Acids Res, 18, 3587, 1990) as well as a p51 expression construct, an HA-labeled p51 expression construct (HA-labeled

- 5 ATGTATCCATATGATGTTCCAGATTATGCT, which codes for the amino acid sequence MYPYDVPDYA), a p53 expression construct, and a vector, and the colony-forming ability was evaluated.

[0294]

- The above expression vectors were constructed by
- 10 cloning the coding region fragment of p51DNA (2816 nucleotides; nucleotide numbers 1-2816), the fragment prepared by adding the HA tag to the p51cDNA, and the coding region fragment of p53cDNA (1698 nucleotides; nucleotide numbers 62-1760), respectively. Then, the
- 15 osteosarcoma cell line SAOS2 was cultured in Dulbeccos's Modified Essential Medium supplemented with 10% fetal calf serum in a 5% CO₂ environment. A 6 cm dish was seeded with the above SAOS2 cells (1×10⁶ cells/dish) and, after 24 hours, the cells were transfected with a wild-type p51
- 20 expression vector containing the p51cDNA chain (pRcCMV/p51). Similar transformations were carried out using the HA-tagged p51cDNA, the wild-type p53 gene and, as control, the pRcCMV expression vector alone.

[0295]

- 25 Using Mammalian Transfection Kit (Stratagene), 1

μ g of pBABEpuro was introduced into the cells. The resulting cells were fixed and stained with Crystal Violet. The stained colonies were photographed. Each transfection was carried out twice for analyzing colony formation.

5 [0296]

As a result, significant decreases in the number of colonies were observed in the culture dishes of p53 gene-transfected cells. In contrast, in the culture dish of cells treated with the vector alone, growth of a large
10 number of colonies was observed. However, the ability of p51 gene to suppress colony formation was found to have slightly decreased. On the other hand, the HA-tagged p51 gene showed a colony formation-suppressing ability comparable to that of the p53 gene (see a reference
15 photograph).

[0297]

Experimental Example 2 Test of the transcriptional activation function of p51

Since the activity of p53 to arrest cell growth
20 in G1 phase or induce apoptosis was dependent on the transcriptional activation function of p53, a test was carried out to see whether p51 would exhibit such activity.

[0298]

Downstream of the Waf1 promoter, which is known
25 to be controlled by p53 transcriptional activation

- 119 -

function, and RGC (ribosomal gene cluster) sequence, a luciferase reporter plasmid as well as a p51 gene expression construct was introduced by the method described in Example 5. Specifically, SAOS2 cells were co-transfected with said luciferase reporter plasmid and the p51 expression vector, p53 expression vector or control vector and the luciferase activity of the lysate obtained from the resulting transformant was assayed. The luciferase activity was calculated with a dual luciferase reporter assay system (ProMega) taking the transfection efficiency into consideration.

[0299]

Fig. 14 is a schematic diagram showing the reporter construct used in the experiment.

15 **[0300]**

Shown in the diagram are 3 luciferase gene constructs each linked downstream of various p21WAF1 promoters. In the diagram, "WAF-1 promoter Iuc" represents a wild-type p21WAF1 promoter construct retaining the two p53 control elements; "del 1" represents the construct deprived of the upstream one of said elements; and "del 2" represents the construct deprived of both of said elements.

[0301]

25 The results are shown in Fig. 15 and Fig. 16.

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Relative activity on the ordinate represents the luciferase activity calculated by using the dual luciferase reporter assay system taking the transfection efficiency into consideration.

5 [0302]

Fig. 15 shows the transactivation activity found when various reporter constructs shown in Fig. 14, the p51 expression plasmid (p51), the p53 expression plasmid (p53) or the vector (Rc/cMV) only was introduced into SAOS2
10 cells. The results showed that like p53, p51 has activity to induce the number-dependent expression of the p53 reactive sequence.

[0303]

Fig. 16 shows the results of a similar
15 experiment using the p51 expression plasmid (p51), the HA-tagged p51 expression plasmid (Hap51), p53 expression plasmid (p53) or the vector (RcCMV), together with the PGC reporter construct which has been experimentally demonstrated to have p53 reactivity. As in the experiment
20 shown in Fig. 14, the above results indicated that both p51 and HAP51, like p53, have activity to induce the number-dependent expression of the p53 reactive sequence. The weak activity found when the p51 expression plasmid was used may be attributed to the fact that since this
25 plasmid was built into the expression vector with the

leader sequence retained, the amount of expression was small.

[0304]

When the leader sequence was removed in a later
5 experiment, p51 showed a stronger expression-inducing
action than p53 and, in said colony formation-inhibition
assay, too, this protein was found to have strong activity.

[0305]

It is apparent from the above results that the
10 p51 protein had the ability to induce transcription
through its transcriptional regulation domain. The
finding that the transcriptional activity was lost on
induced mutation of this element suggests that p51 also
utilizes the same recognition sequence as does p53.

15 [0306]

Then, it was inquired whether the same
transcription relation holds true in vivo, too. A p51
gene expression construct having an HA-tagged epitope was
introduced into SAOS2 cells over a short time. The
20 finding of the uptake of the p51 gene by cells indicated
that p51 is localized in the nucleus and all the cells
were found to elevate the level of p21Waf1. This
indicates that p51 is also capable of inducing p21Waf1
which is known to be controlled by p53.

25 [0307]

Experimental Example 3 p51 gene mutation in tumors in situ

The mutation of the p51 gene was investigated in the in situ cancer cells of 66 patients (neuroblastoma, 8 cases; colon cancer, 8 cases; breast cancer, 8 cases; lung cancer, 8 cases; brain tumor, 8 cases; esophagus cancer, 8 cases, hepatocytoma, 8 cases; pancreatic cancer, 6 cases, and renal cancer, 4 cases) by the reverse transcription PCR single-stranded structure polymorphism (RT-PCR-SSCP) method and DNA sequencing method.

[0308]

(1) Preparation of RNA

Fresh tumor samples were surgically isolated, immediately frozen and stored at -80°C until used. The RNA was extracted by the method described in the report of Nakagawa et al. [Nakagawa, A., et al., N. Engl. J. Med., 328, 847-854 (1993)].

(2) RT-PCR-SSCP and DNA sequencing

The total RNA, 5 μ g, was transcribed on cDNA using Superscript II reverse transcriptase (Gibco-BRL) and random primers. The 20th cDNA of this reaction product was used for PCR amplification. PCR-SSCP was performed in accordance with the method of Mashiyama et al. [Mashiyama S. et al., Oncogene, 6, 1313-1318 (1991)]. Specifically, the PCR product was amplified using 3 primers for p51 cDNA.

the separation was carried out on 5% glycerol/5% polyacrylamide gel at 200 volts at room temperature for 12-14 hours. After the electrophoresis, the gel was dried and exposed against X-ray film overnight so that the migration bands would be definitely visible. To confirm the presence or absence of mutation, the PCR product was

subcloned into the pGEM-T Easy Vector (Promega), followed by sequencing with ABI377 DNA sequencer.

[0311]

As a result, in the lung cancer tissue belonging to the type of highly differentiated squamous cell cancer, an amino acid substitution point mutation (of Ala145 into Pro) was found in the deduced DNA binding region of p51. This tumor showed paratracheal lymphnode metastasis and pleural invasion. Since all of randomly selected 5 clones had the same mutation, the p51 gene possessed by this tumor cell was suggested to be a single allele or have been expressed monoallelically.

[0312]

Experimental Example 4 Induction of apoptosis by p51cDNA introduction

It was explored whether, like the p53 protein, the p51 protein would induce cell apoptosis.

[0313]

The apoptosis induction test with the p51 protein was carried out by the method reported by the present inventors before, namely, the method which comprises the use of a transgenic mouse erythroleukemia cell line (1-2-3 cell line) which presents with typical features of apoptosis when cultured at 32°C [Kato, M. V., et al., Int. J. Oncol., 9, 269 (1996)].

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[0314]

This mouse erythroleukemia cell line (1-2-3 cell line) was established from the erythroleukemia derived from Friend spleen focus forming virus gp55 gene-
5 transgenic mice [Xu et al., Jpn. J. Cancer Res. 86, 284-291 (1995); Kato et al., Int. J. Oncol. 9, 269-277] and is a cell strain which expresses only a temperature-sensitive (ts) mutant p53 protein (mutant p53 protein (Ala1353Val: point mutation): ts-p53). This ts-p53 protein is
10 localized in the cytoplasm at 37°C, which is a usual culture temperature, and, therefore, does not exhibit the control function of the p53 molecule which is intrinsically discharged in the nucleus but at 32°C it migrates into the nucleus so that the p53 activity is
15 induced [Levine, A. J. et al., Nature 351, 453-456 (1991)]. It has been already reported that, in this cell line, slow apoptosis is induced at 32°C.

[0315]

The 1-2-3 cells were cultured in RPMI 1640
20 medium supplemented with 10% fetal calf serum in a 5% CO₂ environment. Then, using pRc/CMV as the expression vector, the p51 gene was introduced into the above cells. The cells were then cultured in a selective medium, and using neomycin resistance (Neor) as the test, G418-resistant
25 cells were selected. An apoptosis induction study was

then carried out in the p51-expressing cells.

[0316]

Thus, two strains of p51-transfected 1-2-3 cells (hereinafter referred to as "1C1 cells" and "4B1 cells") as transfected with the p51 gene-harboring expression vector (pRcCMV/p51) and, as control, 1-2-3 cells transformed with the vector alone and not containing the p51 gene were respectively seeded on 10 cm (dia.) plates at a concentration of 1×10^5 /ml and cultured at 2 alternative temperatures of 37°C and 32°C for 24 hours. The cells were then harvested and treated with proteinase K and Rnase A to prepare DNA samples. The DNA samples were subjected to agarose electrophoresis. The ethidium bromide-stained images are shown in Fig. 17.

15 [0317]

As can be seen in Fig. 17 that, in culture at 37°C, whereas no DNA fragment was detected in 1-2-3 cells (lane 1), DNA fragmentation to 180 bp oligomers could be detected in the p51 gene-transfected 1C1 and 4B1 cells (lanes 2 and 3).

[0318]

In culture at 32°C, DNA fragmentation was detected in 1-2-3 cells (lane 4) and the DNA fragmentation was promoted in 1C1 cells and 4B1 cells (lanes 5 and 6).
25 These results were consonant with the results of

morphological observation of apoptosis and the results of the growth inhibition test of p51-introduced cells (32°C, 37°C).

[0319]

5 The presence or absence of apoptotic morphological changes in cells was studied by fixing the respective cells on glass slides and, after Gimsa staining, observing the cell morphology and the degree of staining microscopically. The viable count of cells was found by
10 Trypan Blue staining and counting.

[0320]

As a result, the cells grown at 32°C had surface projections and presented with a shrunken, strained or constricted form. Moreover, in Gimsa-stained cell
15 specimens, chromatin condensation was observed either around the nuclear envelope or in intracellular masses. In contrast, in the cells cultured at 37°C, no such morphological change was observed.

[0321]

20 Within 24 hours of culture at 32°C, cells undergoing apoptotic death and cells continuing the cell cycle and growth were mixedly present, and after 24 hours the viable cell count of p51-expressing cells was $10^5/\text{ml}$ and the cell count of 1-2-3 cells was $1.7 \times 10^5/\text{ml}$.

25

[0322]

The foregoing indicated that the p51 gene-containing cells treated at the temperature of 32°C experienced a sudden apoptosis in the presence of p53. It was thus confirmed that the p51 protein, like the p53 protein, induces apoptosis in a significant measure.

[Brief Description of the Drawings]

[Fig. 1] Fig. 1 is a diagram showing the amino acid sequence of the human p51 protein of the invention.

[Fig. 2] Fig. 2 is a diagram showing the nucleotide sequence identified by nucleotide numbers 1-400 of the human p51 gene of the invention.

[Fig. 3] Fig. 3 is a diagram showing the nucleotide sequence identified by nucleotide numbers 401-800 of the human p51 gene of the invention.

[Fig. 4] Fig. 4 is a diagram showing the nucleotide sequence identified by nucleotide numbers 801-1200 of the human p51 gene of the invention.

[Fig. 5] Fig. 5 is a diagram showing the nucleotide sequence identified by nucleotide numbers 1201-1600 of the human p51 gene of the invention.

[Fig. 6] Fig. 6 is a diagram showing the nucleotide sequence identified by nucleotide numbers 1601-2000 of the human p51 gene of the invention.

[Fig. 7] Fig. 7 is a diagram showing the nucleotide sequence identified by nucleotide numbers 2001-2400 of the

human p51 gene of the invention.

[Fig. 8] Fig. 8 is a diagram showing the nucleotide sequence identified by nucleotide numbers 2401-2816 of the human p51 gene of the invention.

5 [Fig. 9] Fig. 9 is a diagram showing the homology relationship of the amino acid sequence encoded by the human p51 gene of the invention, the amino acid sequence of the p53 protein, and the amino acid sequence of the p73 β protein. The amino acids which are common among the
10 three sequences are indicated in blocks.

[Fig. 10] Fig. 10 is a diagram illustrating features of the structural domains of the p51 protein, along with those of the p53 protein and p73 β protein. In the diagram, "TA" represents a transcriptional activator domain; "DNA
15 binding" represents a DNA binding domain; and "Oligo" represents an oligomerization domain.

[Fig. 11] Fig. 11 is a photograph, in lieu of a drawing, which shows the pattern of expression of p51mRNA in various human tissues as a Northern blotting (using a
20 Clontech's filter) electrophoretogram. The lanes represent the results for 1: heart, 2: brain, 3: placenta, 4: lung, 5: liver, 6: skeletal muscle, 7: spleen, 8: pancreas, respectively.

[Fig. 12] Fig. 12 is a photograph, in lieu of a drawing,
25 which shows the pattern of expression of p51mRNA in

various human tissues as a Northern blotting (using a filter prepared by using the RNA purchased from Clontech) electrophoretogram. The lanes represent the results for 1: mammary gland, 2: prostate, 3: salivary gland, 4: stomach, 5: thymus, 6: thyroid, 7: trachea, and 8: uterus, respectively.

[Fig. 13] Fig. 13 is a schematic diagram comparing the structure of the alternative splicing variant (A, B) of the p51 protein with the structure of the alternative splicing variant (α , β) of the p73 protein.

[Fig. 14] Fig. 14 is a schematic diagram showing the reporter constructs used in Experimental Example 2. In the diagram, "WAF-1 promoter luc" represents a wild type p21WAF1 promoter construct with two p53 regulating elements retained; "del 1" represents a similar construct in which one upstream element has been deleted; and "del 2" represents a construct in which both elements have been deleted.

[Fig. 15] Fig. 15 shows the transactivation activity found when the p51 expression plasmid (p51), the p53 expression plasmid (p53) or the vector (Rc/CMV) only, which has been linked to each of the various reporter constructs shown in Fig. 14, was introduced into SAOS2 cells (Experiment Example 2).

[Fig. 16] Fig. 16 shows the results of an experiment

In the diagram, the "1-2-3 cells" represents control cells into which the vector only has been introduced and not containing the p51 gene and the "1C1 cells" or "4B1 cells" represents the p51-containing 1-2-3 cells as transformed with the expression vector harboring the p51 gene (pRcCMV/p51A). The " λ /Hind III" represents digestion products as digested with the λ phage DNA restriction enzyme Hind III and are DNA size markers (product of New England Biolabs. Ind.). The "100 bp ladder" represents size markers comprising DNA fragments having sizes corresponding to multiples of 100 bp (product of GIBCO-BRL).

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[Document Name] Document describing chemical formulas,
etc.

Specification

[Table 1]

	Full-length sequence	Transcription activation domain	DNA binding domain	Oligomerization domain
p51→p53	36%	22%	60%	37%
p51→ p73	42%	30%	87%	65%
p53 →p73	28%	27%	63%	83%

[Fig. 2]

TCGTTGATAT CAAAGACAGT TGAAGGAAAT GAATTTTGAA ACTTCACGGT 50
S L I S K T V E G N E F X N F T V

GTGCCACCCT ACAGTACTGC CCTGACCCTT ACATCCAGCG TTTGCTAGAA 100
C H P T V L P X P L H P A F R R N

ACCCAGCTCA TTTCTCTTGG AAAGAAAGTT ATTACCGATC CACCATGTCC 150
P A H F S W K E S Y Y R S T M S

CAGAGCACAC AGACAAATGA ATTCCTCAGT CCAGAGGTTT TCCAGCATAT 200
Q S T Q T N E F L S P E V F Q H I

CTGGGATTTT CTGGAACAGC CTATATGTTT AGTTCAGCCC ATTGACTTGA 250
W D F L E Q P I C S V Q P I D L N

ACTTTGTGGA TGAACCATCA GAAGATGGTG CGACAAACAA GATTGAGATT 300
F V D E P S E D G A T N K I E I

AGCATGGACT GTATCCGCAT GCAGGACTCG GACCTGAGTG ACCCCATGTG 350
S M D C I R M Q D S D L S D P M W

GCCACAGTAC ACGAACCTGG GGCTCCTGAA CAGCATGGAC CAGCAGATTC 400
P Q Y T N L G L L N S M D Q Q I Q

[Fig. 4]

TGCCCCCTCT AGTCATTTGA TTGAGTAGA GGGGAACAGC CATGCCAGT 850
A P P S H L I R V E G N S H A Q Y

ATGTAGAAGA TCCCATCACA GGAAGACAGA GTGTGCTGGT ACCTTATGAG 900
V E D P I T G R Q S V L V P Y E

CCACCCAGG TTGGCACTGA ATTCACGACA GTCTTGACA ATTCATGTG 950
P P Q V G T E F T T V L Y N F M C

TAACAGCAGT TGTGTTGGAG GGATGAACCG CCGTCCAATT TTAATCATTG 1000
N S S C V G G M N R R P I L I I V

TTACTCTGGA AACCAGAGAT GGGCAAGTCC TGGGCCGACG CTGCTTTGAG 1050
T L E T R D G Q V L G R R C F E

GCCCGGATCT GTGCTTGCCC AGGAAGAGAC AGGAAGGCGG ATGAAGATAG 1100
A R I C A C P G R D R K A D E D S

CATCAGAAAG CAGCAAGTTT CGGACAGTAC AAAGAACGGT GATGGTACGA 1150
I R K Q Q V S D S T K N G D G T K

AGCGCCCGTT TCGTCAGAAC ACACATGGTA TCCAGATGAC ATCCATCAAG 1200
R P F R Q N T H G I Q M T S I K

[Fig. 5]

AAACGAAGAT CCCAGATGA TGAAGTGTGA TACTTACCAG TGAGGGGCCG 1250
K R R S P D D E L L Y L P V R G R

TGAGACTTAT GAAATGCTGT TGAAGATCAA AGAGTCCCTG GAACTCATGC 1300
E T Y E M L L K I K E S L E L M Q

AGTACCTTCC TCAGCACACA ATTGAAACGT ACAGGCAACA GCAACAGCAG 1350
Y L P Q H T I E T Y R Q Q Q Q Q

CAGCACCAGC ACTTACTTCA GAAACATCTC CTTTCAGCCT GCTTCAGGAA 1400
Q H Q H L L Q K H L L S A C F R N

TGAGCTTGTG GAGCCCCGGA GAGAACTCC AAAACAATCT GACGTCTTCT 1450
E L V E P R R E T P K Q S D V F F

TTAGACATTC CAAGCCCCCA AACCGATCAG TGTACCCATA GAGCCCTATC 1500
R H S K P P N R S V Y P X S P I

TCTATATTTT AAGTGTGTGT GTTGTATTTC CATGTGTATA TGTGAGTGTG 1550
S I F X V C V L Y F H V Y M X V C

TGTGTGTGTA TGTGTGTGCG TGTGTATCTA GCCCTCATAA ACAGGACTTG 1600
V C V C V C V C I X P S X T G L E

[Fig. 6]

AAGACACTTT GGCTCAGAGA CCCAACTGCT CAAAGGCACA AAGCCACTAG 1650
D T L A Q R P N C S K A Q S H X

TGAGAGAATC TTTTGAAGGG ACTCAAACCT TTACAAGAAA GGATGTTTTC 1700
X E N L L K G L K P L Q E R M F S

TGCAGATTTT GTATCCTTAG ACCGGCCATT GGTGGGTGAG GAACCACTGT 1750
A D F V S L D R P L V G E E P L C

GTTTGTCTGT GAGCTTTCTG TTGTTTCCTG GGAGGGAGGG GTCAGGTGGG 1800
L S V S F L L F P G R E G S G G

GAAAGGGGCA TTAAGATGTT TATTGGAACC CTTTCTGTC TTCTTCTGTT 1850
E R G I K M F I G T L F C L L L L

GTTTTTCTAA AATTCACAGG GAAGCTTTTG AGCAGGTCTC AAACCTAAGA 1900
F F X N S Q G S F X A G L K L K M

TGTCTTTTTA AGAAAAGGAG AAAAAAGTTG TTATTGTCTG TGCATAAGTA 1950
S F X E K E K K V V I V C A X V

AGTTGTAGGT GACTGAGAGA CTCAGTCAGA CCCTTTTAAT GCTGGTCATG 2000
S C R X L R D S V R P F X C W S C

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[Fig. 7]

TAATAATATT GCAAGTAGTA AGAAACGAAG GTGTCAAGTG TACTGCTGGG 2050
N N I A S S K K R R C Q V Y C W A

CAGCGAGGTG ATCATTACCA AAAGTAATCA ACTTTGTGGG TGGAGAGTTC 2100
A R X S L P K V I N F V G G E F

TTTGTGAGAA CTTGCATTAT TTGTGTCCTC CCCTCATGTG TAGGTAGAAC 2150
F V R T C I I C V L P S C V G R T

ATTTCTTAAT GCTGTGTACC TGCCTCTGCC ACTGTATGTT GGCATCTGTT 2200
F L N A V Y L P L P L Y V G I C Y

ATGCTAAAGT TTTTCTTGTA CATGAAACCC TGGAAAGACCT ACTACAAAAA 2250
A K V F L V H E T L E D L L Q K

AACTGTTGTT TGGCCCCCAT AGCAGGTGAA CTCATTTTGT GCTTTTAATA 2300
N C C L A P I A G E L I L C F X X

GAAAGACAAA TCCACCCCAG TAATATTGCC CTTACGTAGT TGTITACCAT 2350
K D K S T P V I L P L R S C L P L

TATTCAAAGC TCAAAATAGA ATTTGAAGCC CTCTCACAAA ATCTGTGATT 2400
F K A Q N R I X S P L T K S V I

[Fig. 8]

AATTTGCTTA ATTAGAGCTT CTATCCCTCA AGCCTACCTA CCATAAAACC 2450
N L L N X S F Y P S S L P T I K P

AGCCATATTA CTGATACTGT TCAGTGCATT TAGCCAGGAG ACTTACGTTT 2500
A I L L I L F S A F S Q E T Y V L

TGAGTAAGTG AGATCCAAGC AGACGTGTGA AAATCAGCAC TCCTGGACTG 2550
S K X D P S R R V K I S T P G L

GAAATTAAG ATTGAAAGGG TAGACTACTT TTCTTTTTTT TACTCAAAG 2600
E I K D X K G R L L F F F L L K S

TTTAGAGAAT CTCTGTTTCT TTCCATTTTA AAAACATATT TTAAGATAAT 2650
L E N L C F F P F X K H I L R X X

AGCATAAAGA CTTTAAAAAT GTTCCTCCCC TCCATCTTCC CACACCCAGT 2700
H K D F K N Y P P L H L P T P S

CACCAGCACT GTATTTTCTG TCACCAAGAC AATGATTTCT TGTTATTGAG 2750
H Q H C I F C H Q D N D F L L L R

GCTGTGCTT TTGTGGATGT GTGATTTTAA TTTTCAATAA ACTTTTGCAT 2800
L L L L W M C D F N F Q X T F A S

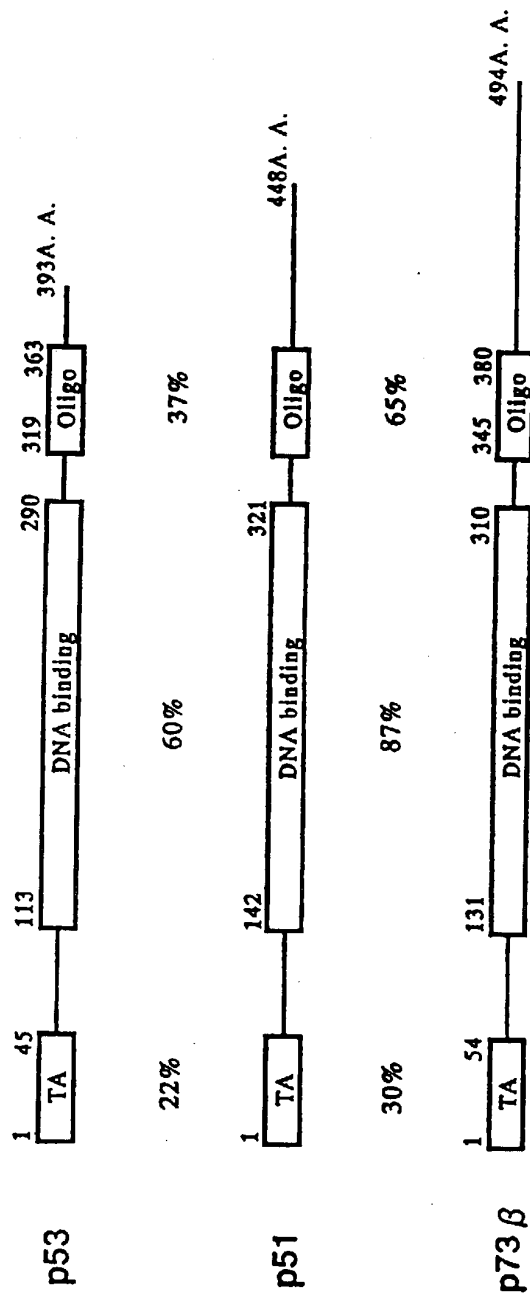
CTTGQTITAA AAGAAA 2816
W F K R

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[Fig. 9]

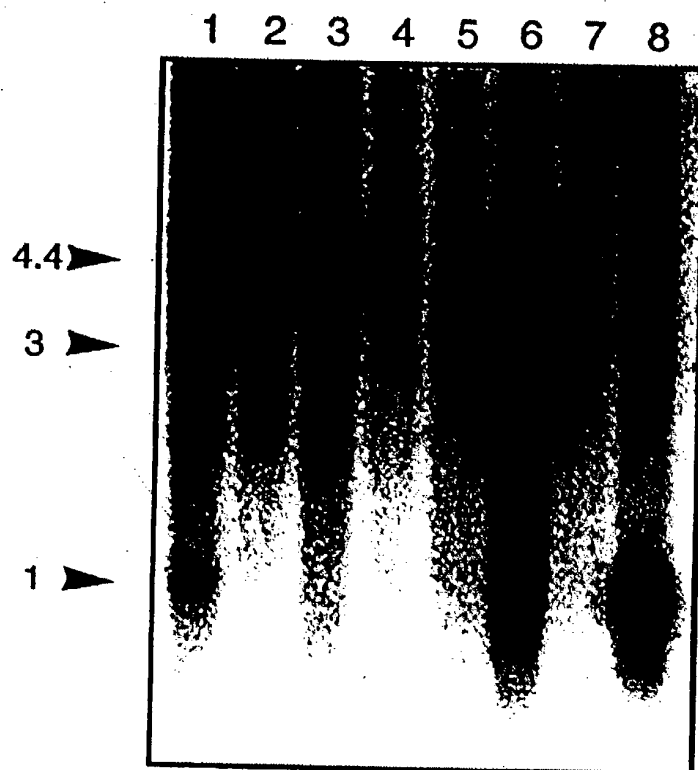
p53	RETPQSDPS- VEPPLSQETI SLL-----WMD---L FENNVLSPLP	36
p51	REGSTQTHY LGEVTEQHW DLEQFICSV QPILNPFVDE PSBDGATNKI	50
p73b	HAOSTATGP- DGGTTTFLHW SLL-PDSTY --FLLP-QSS RGNNEVVGCT	45
Consensus	RE GST.T... ..P.FGR.W S.LL-P.... --CL.... P.NH.....	50
p53	SQANDDLMS ---PDDI-E QN---PTED PGFDEAPRMP EARR-RVARR	76
p51	EISNDGIRNQ DSDLSDFMWP QYTLGLLNS MDQIQNGSS STEFYNTDHA	100
p73b	DSSNDVPLEH --GMTTSVMA QY---NLLSS TMDQSSRAA SASFTPTERR	90
Consensus	..SND...L.D.... Q---.LL.S ...Q...R.. SASFY...HA	100
p53	-PAAPITAA- PAREPHW-PL SSS--VPSCK TYGSGYGLR CLHSSTAKG	121
p51	QNEVTAPSYI ACESSTFDAL SPSPAPPEPT DYSGPHSDV SDQCSSTAKG	150
p73b	-ASVITESPY ACESSTFDTH SPAPVPEPT DYSGPHSEV TYQCSSTAKG	139
Consensus	-.SVITPSFY ACESSTFD.L SPSP.PEPT DYSGP.H.V .QDSSSTAKG	150
p53	VITYSPLIN KMFQIAKTC PQQNVDSIF PEGTARARA TYQDSCHTE	171
p51	MYATSTELK KLVQIAKTC PQQNVMTHE PGCAVIRAMP VYKCAEATE	200
p73b	MYATSPILK KLVQIAKTC PQQNVSTEF PEGTARARA VYKCAEATE	189
Consensus	MYATSP.LK KLVQIAKTC PQQNV.THE PEGTARARA VYKCAEATE	200
p53	VYRCFPHRR CSQ-SQC-DA PQCHLKVEG NLRVETLDR NTFRRSVNVG	219
p51	VYRCFPHRL SRETFEGDA PSHLLKVEG NHAQVDEP LTRGVNVG	250
p73b	VYRCFPHRL GRDFEGQSA PASHLKVEG NNLSCVLEP VTRGVNVG	239
Consensus	VYRCFPHRL .RDFEGQ .A PSHLLKVEG N...CVLEP LTRGVNVG	250
p53	VEPPVGSDC ITTHNMCMV SSCGGMNR PILLITLTD SSCNVLGRNS	269
p51	VEPPVGTET ITTHNMCMV SSCGGMNR PILLITLET RDSVVLGRRC	300
p73b	VEPPVGTET ITTHNMCMV SSCGGMNR PILLITLTM RDSVVLGRRS	289
Consensus	VEPPVGTET ITTHNMCMV SSCGGMNR PILLITL.. RDSVVLGRRS	300
p53	FEARACACG RDRALDESL RKQGEPR--K ELPPGATRA LPNRTSS---	314
p51	FEARACACG RDRALDESI RKQGVSD--S TYNGDGTARP FRONTHGI-Q	347
p73b	FEARACACG RDRALDEHY RKQALNESH ANGAAGTRA FKQSPRAVPA	339
Consensus	FEARACACG RDRALDE.. RKQ.....S .XNG..TRA F.QNT....	350
p53	--SPQHKKP IDGHTTLOI RGRERHETR KINELELKI AQACKEPGGS	362
p51	M-TSINKRRS HDELLYLPV RGRERTHLL KINELELKI YLPQNTIETI	396
p73b	LQAGVETRRH QDSHTYLOV RGRERHETL KINELELME LVPQPLVDSY	389
ConsensusKRR. .H.E..YLOV RGR..HETL. KINELELM. .PQ.....Y	400
p53	RAESS-----HL-----K-----SKKQ-----STSRH--	380
p51	RDQDQDQHQ--NI-----LCK-H--LLSACPRNE LV-EPRRE--	427
p73b	RDQDQLLQRP SELLPPSYGP VLSYMKVHG GHNKLPSVNO LVGQPPHSS	439
Consensus	RDQDQ....HL-----K-H-----NQ LV-.P.RH--	450
p51	---KKL--- --MFK---T EGPD-----SD---	393
p51	---TPKQSDV-- --FTR---H KPPH-----RSVYP-	448
p73b	AATPNLGFVG PGMNNHCHA VPANGHSSS HSAQSHVSGS RCTPPPYHA	489
Consensus	---TPKL..V- --MP---H..PFFH-----..S.Y.-	500
p53	-----	393
p51	-----	448
p73b	DPSLVRTWGP	499
Consensus	-----	510

[Fig. 10]



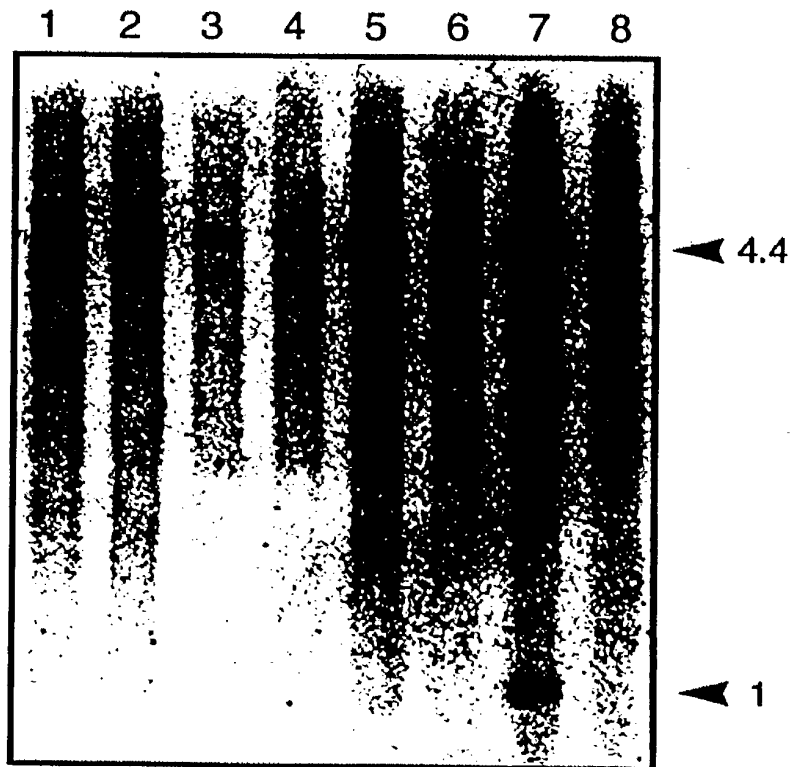
-143-

[Fig. 11]

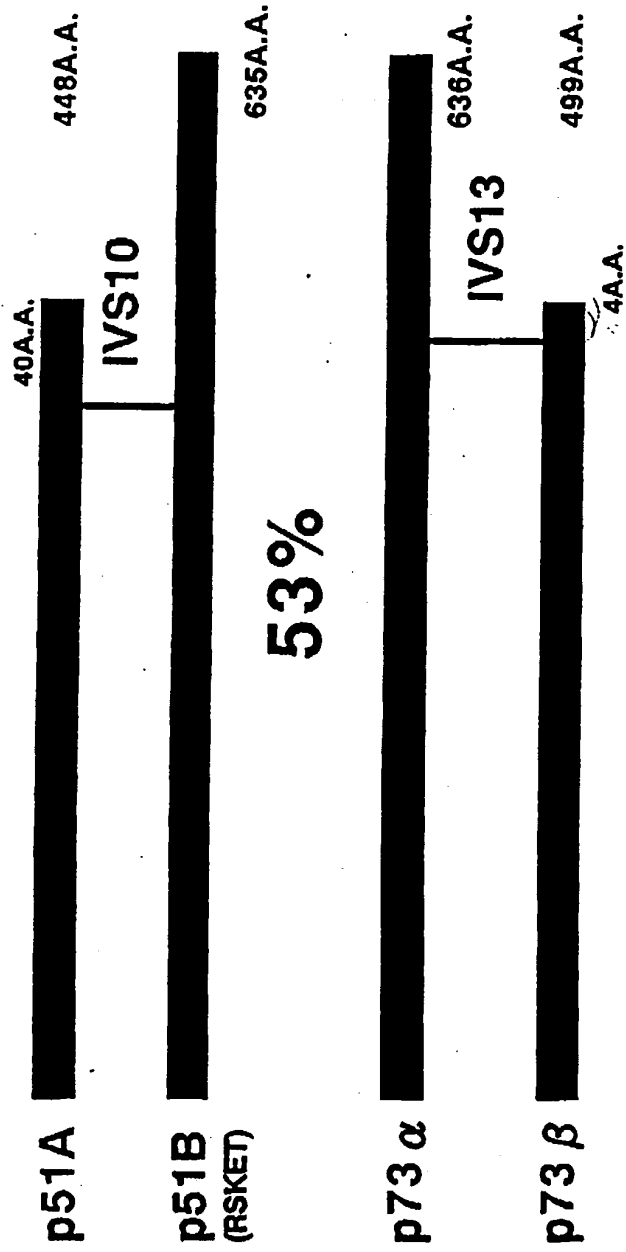


-144-

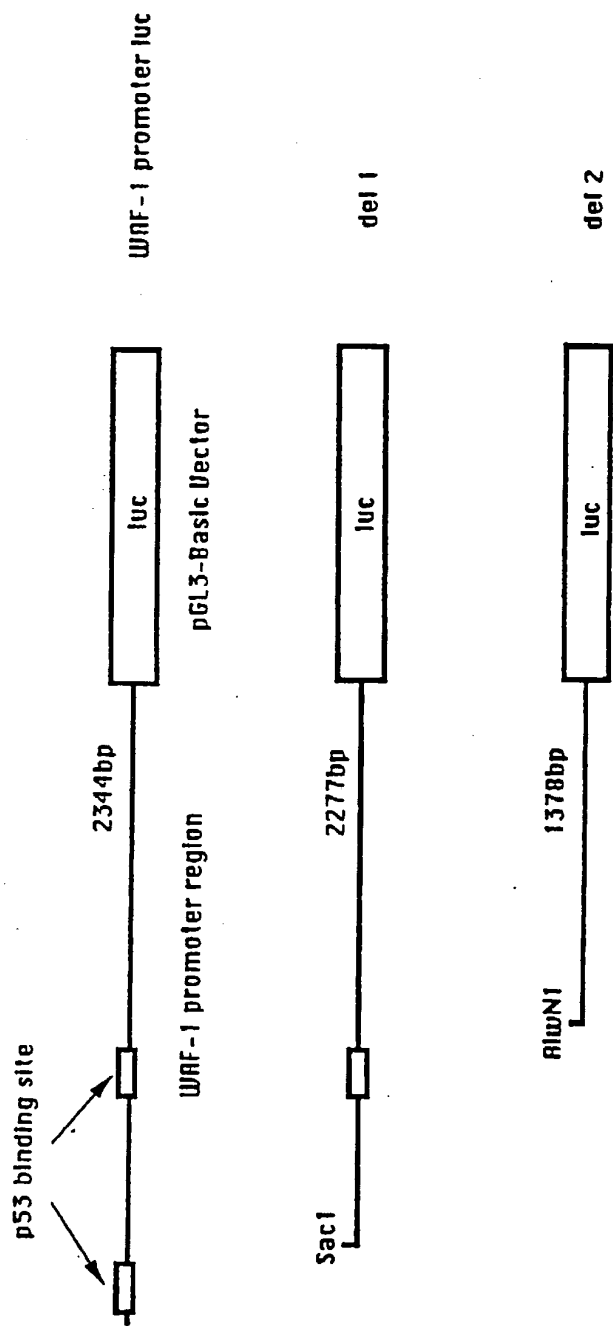
[Fig. 12]



[Fig. 13]



[Fig. 14]



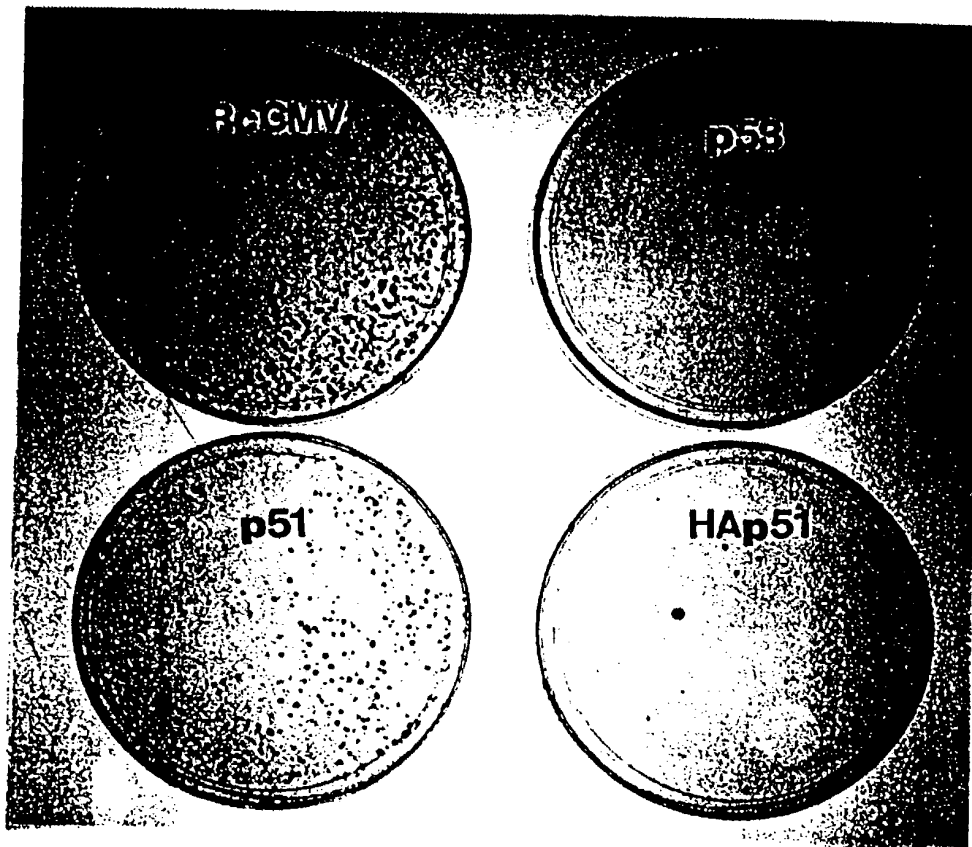






-150-

[Reference Photograph]



[Document Name] Abstract

[Abstract]

[Object] Provision of a novel human gene which
is included in the family of p53 gene known as a cell
5 proliferation inhibitory gene, and gene products thereof.

[Method for Achieving the Object] A human p51
gene comprising the nucleotide sequence coding for the
amino acid sequence shown in Fig. 1; a human p51 gene
comprising comprising a nucleotide sequence identified by
10 the nucleotide numbers 145-1487 of the nucleotide sequence
shown in Figs. 2-8; a vector harboring the gene; a host
cell transformed with the vector; a method of producing a
p51 protein having the amino acid sequence shown in Fig. 1,
which comprises growing the host cell and harvesting a
15 protein from the resulting culture; and the p51 protein.

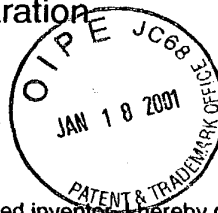
[Selected Figure] None

Declaration and Power of Attorney for Patent Application

特許出願宣言書及び委任状

Japanese Language Declaration

日本語宣言書



下記の氏名の発明者として、私は以下の通り宣言します。

As a below named inventor, I hereby declare that:

私の住所、私書箱、国籍は下記の私の氏名の後に記載された通りです。

My residence, post office address and citizenship are as stated next to my name,

下記の名称の発明に関して請求範囲に記載され、特許出願している発明内容について、私が最初かつ唯一の発明者(下記の氏名が一つの場合)もしくは最初かつ共同発明者であると(下記の名称が複数の場合)信じています。

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

HUMAN p51 GENES AND GENE PRODUCTS THEREOF

上記発明の明細書(下記の欄でX印がついていない場合は、本書に添付)は、

the specification of which is attached hereto unless the following box is checked:

☐ ____月 ____日に提出され、米国出願番号または特許協定条約

☒ was filed on September 27, 2000
as United States Application Number or
PCT International Application Number

国際出願番号を ____ とし、

09/670568 and was amended on

(該当する場合) ____ に訂正されました。

____ (if applicable).

私は、特許請求範囲を含む上記訂正後の明細書を検討し、内容を理解していることをここに表明します。

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

私は、連邦規則法典第37編第1条56項に定義されるとおり、特許資格の有無について重要な情報を開示する義務があることを認めます。

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

Japanese Language Declaration (日本語宣言書)

私は、米国法典第35編第119条(a)-(d)項又は第365条(b)項に基づき下記の、米国以外の国の少なくとも一カ国を指定している特許協力条約第365条(a)項に基づく国際出願、又は外国での特許出願もしくは発明者証の出願についての外国優先権をここに主張するとともに、優先権を主張している本出願の前に出願された特許または発明者証の外国出願を以下に、枠内をマークすることで、示しています。

Prior Foreign Applications

外国での先行出願

1998-100467	Japan
(Number) (番号)	(Country) (国名)
(Number) (番号)	(Country) (国名)
(Number) (番号)	(Country) (国名)

私は、第35編米国法典119条(e)項に基づいて下記の米国特許出願規定に記載された権利をここに主張致します。

(Application No.) (出願番号)	(Filing Date) (出願日)

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(Application No.) (出願番号)	(Filing Date) (出願日)
(Application No.) (出願番号)	(Filing Date) (出願日)

私は、私自身の知識に基づいて本宣言中で私が行う表明が真実であり、かつ私の入手した情報と私の信ずるところに基づく表明が全て真実であると信じていること、さらに故意になされた虚偽の表明及びそれと同等の行為は米国法典第18編第1001条に基づき、罰金または拘禁、もしくはその両方により処罰されること、そしてそのような故意による虚偽の声明を行えば、出願した、又は既に許可された特許の有効性が失われることを認識し、よってここに上記のごとく宣誓を致します。

I hereby claim foreign priority under Title 35, United States Code, Section 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Priority Not Claimed

優先権主張なし

27/03/1998	<input type="checkbox"/>
(Day/Month/Year Filed) (出願年月日)	
	<input type="checkbox"/>
(Day/Month/Year Filed) (出願年月日)	
	<input type="checkbox"/>
(Day/Month/Year Filed) (出願年月日)	

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

(Application No.) (出願番号)	(Filing Date) (出願日)

I hereby claim the benefit of Title 35, United States Code Section 120 of any United States application(s), or 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code Section 112, I acknowledge the duty to disclose any material information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Status: Patented, Pending, Abandoned) (現況: 特許許可済、係属中、放棄済)
(Status: Patented, Pending, Abandoned) (現況: 特許許可済、係属中、放棄済)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Japanese Language Declaration

(日本語宣言書)

委任状: 私は、下記の発明者として、本出願に関する一切の手続きを米国特許商標局に対して遂行する弁理士又は代理人として、下記のものを指名致します。(弁理士、又は代理人の氏名及び登録番号を明記のこと)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith (list name and registration number)

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Japanese Language Declaration (日本語宣言書)

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国籍		Citizenship	
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住所		Residence	
国籍		Citizenship	
郵便の宛先		Post office address	